OUTI KONTKANEN

Gene expression in rat brain: alterations by antipsychotic drugs

Doctoral dissertation

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Department of Neurobiology
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University of Kuopio

KUOPIO YLIOPISTO
KUOPIO 2002
Antipsychotic drug treatment is a cornerstone therapy in treatment of schizophrenia. The prototype typical antipsychotic drug haloperidol is the most commonly used antipsychotic, which may lead to appearance of extrapyramidal symptoms (EPS) in chronic administration. Clozapine, the archetype atypical antipsychotic drug, induces fewer EPS and is efficient for patients responding poorly to typical antipsychotic drugs. Antipsychotic drugs bind neurotransmitter receptors immediately after administration, whereas their clinical efficacy is achieved after weeks of medication. Alterations in gene expression are likely to occur during the adaptation period. The prefrontal cortex (PFC) has been suggested as potentially involved in schizophrenic abnormalities, and in the function of antipsychotic drugs. In this study, the expression of fos and jun family transcription factors, and other candidate target genes was examined following antipsychotic drug treatments in a cell culture model and in rat brain. In addition, effects of MK-801, a non-competitive NMDA-receptor antagonist and a psychotroic drug, were studied on fos and jun family gene expression in the entorhinal cortex.

Acute MK-801 treatment induced fos and jun family gene mRNAs in the rat entorhinal cortex in a layer-specific manner. The DNA binding activity of the activator protein 1 (AP-1) complex formed by Fos and Jun proteins was also increased, suggesting functional changes in gene expression by MK-801.

Altered expression of fos and jun family gene mRNAs was observed in several brain regions after acute (1h) haloperidol and clozapine treatments. Chronic treatments (17d) with various washout periods (2h, 24h, and 6d) revealed a long-lasting expression of several fos and jun family genes, especially in the PFC. Haloperidol increased the expression after 24h and 6d withdrawal periods, and clozapine after 6d drug-free period. However, the DNA-binding activity of the AP-1 complex was altered only by clozapine. The data supports a role for the PFC in the actions of chronic antipsychotic drug treatments, however, drug withdrawal may play a role in the induction of fos and jun family genes after cessation of the treatments.

A PC12 cell culture model was used to establish DNA array methodology, and differential expression of four candidate genes was confirmed after 5d treatment with chlorpromazine, a typical antipsychotic drug. Cytochrome c oxidase was induced, suggesting increased metabolism. In addition, three unknown genes were found induced by chlorpromazine.

DNA arrays were used to search candidate genes for antipsychotic drug action in vivo after acute clozapine treatment (1, 6, and 24h) in the rat PFC. Data clustering with a self-organizing map algorithm revealed an altered expression of genes related to presynaptic function (PSYN) that was further confirmed. The expression patterns of the candidate genes derived from this analysis were also examined following chronic (17d) haloperidol and clozapine treatments using in situ hybridization. Differential expression of several genes belonging to PSYN category was observed after chronic treatments in the cortex. In addition, genes involved in calcium regulation were differentially expressed by chronic treatments. The data supports the proposed role of PSYN class genes in antipsychotic drug action.

In conclusion, potential candidate target genes were identified for acute and chronic antipsychotic drug treatments. The data is applicable in schizophrenia research and in pharmacological target discovery.
“With years a richer life begins,
The spirit mellows:
Ripe age gives tone to violins,
Wine and good fellows.”

John Townsend Trowbridge
1827-1916
ACKNOWLEDGEMENTS

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Kuopio, May 2002

Outi Kontkanen
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>adenylate cyclase</td>
</tr>
<tr>
<td>Acb</td>
<td>nucleus accumbens</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>ATF</td>
<td>activating transcription factor</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Cg cx</td>
<td>anterior cingulate cortex</td>
</tr>
<tr>
<td>CPu</td>
<td>caudate putamen, striatum</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding protein</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary RNA</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diamine-tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>Fr cx</td>
<td>frontal cortex</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-amino-butyric-acid</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>IEG</td>
<td>immediate early gene</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>phencyclidine</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>PKA</td>
<td>cyclic AMP dependent protein kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>(p-aminophenyl)methanesulfonyl fluoride</td>
</tr>
<tr>
<td>PSYN</td>
<td>presynaptic genes</td>
</tr>
<tr>
<td>RDA</td>
<td>representational difference analysis</td>
</tr>
<tr>
<td>RNA</td>
<td>ribodeoxynucleic acid</td>
</tr>
<tr>
<td>SAGE</td>
<td>serial analysis of gene expression</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulphate</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SOM</td>
<td>self-organizing map</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>TOGA</td>
<td>total analysis of gene expression</td>
</tr>
<tr>
<td>VSNL-1</td>
<td>visinin-like protein 1</td>
</tr>
<tr>
<td>VSNL-2</td>
<td>visinin-like protein 2</td>
</tr>
<tr>
<td>VSNL-3</td>
<td>visinin-like protein 3</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications referred to by their corresponding Roman numerals:


In addition, some unpublished data is presented.
TABLE OF CONTENTS

1 INTRODUCTION 15

2 REVIEW OF THE LITERATURE 17
   2.1 DOPAMINERGIC SYSTEM IN BRAIN 17
   2.2 GLUTAMATERGIC SYSTEM IN BRAIN 18
   2.3 FOS AND JUN FAMILY GENES AND THE AP-1 COMPLEX 20
      2.3.1 INDUCTION AND FUNCTION 20
   2.4 GENE EXPRESSION ANALYSIS IN THE BRAIN 22
      2.4.1 HIGH-THROUGHPUT METHODS FOR GENE EXPRESSION ANALYSIS 22
         2.4.1.1 DNA EXPRESSION ARRAYS 22
         2.4.1.2 DIFFERENTIAL DISPLAY 24
         2.4.1.3 SERIAL ANALYSIS OF GENE EXPRESSION 25
         2.4.1.4 OTHER METHODS 26
   2.5 SCHIZOPHRENIA 27
      2.5.1 BIOLOGY OF SCHIZOPHRENIA 28
         2.5.1.1 DOPAMINE AND SCHIZOPHRENIA 29
         2.5.1.2 GLUTamate AND SCHIZOPHRENIA 30
      2.5.2 GENE EXPRESSION IN SCHIZOPHRENIA 32
   2.6 ANTIPSYCHOTIC DRUGS 33
      2.6.1 TYPICAL ANTIPSYCHOTIC DRUGS 34
      2.6.2 ATYPICAL ANTIPSYCHOTIC DRUGS 35
      2.6.3 GENE EXPRESSION BY ANTIPSYCHOTIC DRUGS 36
         2.6.3.1 FOS AND JUN FAMILY GENE EXPRESSION 36
         2.6.3.2 EXPRESSION OF NEUROTRANSMITTER RECEPTORS AND OTHER GENES 38

3 AIMS OF THE STUDY 41

4 EXPERIMENTAL PROCEDURES 42
   4.1 PC12 CELL CULTURE 42
   4.2 ANIMALS 42
   4.3 DRUG TREATMENTS 43
   4.4 IN SITU HYBRIDIZATION 43
   4.5 IMMUNOHISTOCHEMISTRY 44
   4.6 ELECTROMOBILITY SHIFT ASSAY 45
4.7 Northern Blotting 46
4.8 Expression Arrays Used in the Study 47
4.9 Expression Array Procedures 47
4.10 Expression Array Data Analysis 48
  4.10.1 Data Clustering and Visualization with Self-Organizing
       Map Algorithm 49
4.11 Statistical Analysis 50

5 RESULTS 51
  5.1 MK-801 Induces Fos and Jun Expression and AP-1 Binding in
       the Rat Entorhinal Cortex 51
  5.2 Chlorpromazine Induces Differential Gene Expression
       in PC12 Cells 51
  5.3 Acute Clozapine and Haloperidol Treatments and
       Gene Expression in Rat Brain 52
      5.3.1 Fos and Jun Family Transcription Factors 52
      5.3.2 Differential Gene Expression by Array Analysis 52
  5.4 Chronic Clozapine and Haloperidol Treatments and
       Gene Expression in Rat Brain 53
      5.4.1 Fos and Jun Family Transcription Factors and AP-1
             Complex 53
      5.4.2 Differential Expression of Other Candidate Genes 55

6 DISCUSSION 58
  6.1 The Cortex as a Site of Psychotropic and Antipsychotic
       Drug Action 58
  6.2 Use of Expression Arrays to Study Gene Expression in Brain 59
  6.3 Gene Expression by Acute MK-801 and Antipsychotic
       Drug Treatments 64
  6.4 Gene Expression by Chronic Antipsychotic Drug Treatments 67
  6.5 Applications to Antipsychotic Drug Research and
       Schizophrenia 70

7 SUMMARY 72

8 REFERENCES 73

APPENDIX: ORIGINAL PUBLICATIONS I-IV
1 INTRODUCTION

Rapid progress in genome sequencing projects has enabled investigation of the genetic background of various diseases. In addition to genetic linkage studies, examination of gene expression is currently evolving as among the most widely applied approaches in genome biology and research. Genes determine the properties of any tissue in the body, and variations in their expression levels and patterns have profound outcomes in physiology. However, gene expression is usually functionally meaningful only when the corresponding proteins are formed. Analysis of gene expression levels is thus used for indirect estimation of the functionally active protein amounts. High-throughput methods for gene expression analysis have enabled simultaneous and parallel investigation of expression profiles of thousands of genes (Liang and Pardee, 1992; Schena et al., 1995; Velculescu et al., 1995; Zhao et al., 1995). Gene expression studies using microarrays have greatly advanced the knowledge of cell type and region specific gene expression (Zirlinger et al., 2001), molecular background of disease states (Alizadeh et al., 2000; Perou et al., 1999), and functions of entire organisms (Wodicka et al., 1997).

Along with these efforts, the molecular background of schizophrenia has been recently studied using DNA microarrays (Hakak et al., 2001; Middleton et al., 2002; Mimmack et al., 2002; Mirmics et al., 2000; Vawter et al., 2001). These studies have pinpointed many functionally related gene groups as affected in the disease. Specifically, altered expression of several genes related to synaptic function has been observed (Mirmics et al., 2000; Vawter et al., 2001), and these results are supported by previous single gene studies (Eastwood and Harrison, 2001; Glantz and Lewis, 1997; Webster et al., 2001).

Schizophrenia is a complex neuronal disease that affects approximately 1% of the population (Jablensky et al., 1992). The underlying mechanisms of schizophrenia are unknown, but several hypotheses have been presented, including neurodevelopmental disturbances and abnormal neuronal connectivity (Andreasen, 2000). The limbic cortex among other regions appears disturbed in schizophrenic patients by neurochemical, functional, and morphological measures (Buchanan et al., 1998; Eastwood et al., 2001; Harrison, 1999a; Shenton et al., 2001).

Pharmacological intervention of schizophrenia with antipsychotic drug treatment is required for long periods, often for a lifetime. Antipsychotic drugs have been classified as typical and atypical agents that have different neurochemical and functional properties. Typical antipsychotic drugs, like haloperidol, potently antagonize dopamine D₂ class
receptors. While beneficial for many patients, typical antipsychotics are often accompanied by the development of extrapyramidal side effects after prolonged administration. Atypical antipsychotics, like clozapine, have less affinity to D<sub>2</sub> class receptors, but antagonize dopamine D<sub>1</sub> class receptors, and serotonin 5HT<sub>2</sub> receptors, among many others. Clinically effective doses of clozapine rarely induce extrapyramidal symptoms, are effective towards the negative symptoms of schizophrenia, and alleviate the symptoms in patients non-responsive to typical antipsychotics.

All clinically effective antipsychotic drugs produce their effects slowly, typically after weeks of continuous administration, whereas dopamine receptor antagonism is immediate. Therefore, other factors, such as altered gene expression in response to drug treatment, may be involved (Konradi and Heckers, 2001). Indeed, antipsychotic drugs modulate the expression of immediate early gene transcription factors, dopamine receptors, and synaptic proteins, among many others (Dragunow et al., 1990; Eastwood et al., 2000; Janowsky et al., 1992; Lidow et al., 1998; Nguyen et al., 1992). Moreover, long-term antipsychotic drug administration produces altered neuronal and synaptic morphology in animal models (Harrison, 1999b; Konradi and Heckers, 2001). In addition to striatal effects, many of these findings are manifested in various regions of the limbic cortex, thus suggesting a common site of schizophrenic alterations and antipsychotic drug treatment effects (Harrison, 1999b; Langlois et al., 2001; Merchant and Dorsa, 1993; Pilowsky et al., 1997; Robertson and Fibiger, 1992).

In the present study, the gene expression patterns of immediate early gene transcription factors were investigated in rat brain following acute and chronic administration of antipsychotic drugs haloperidol and clozapine. Furthermore, DNA arrays were utilized to search for potential target genes for antipsychotic drug treatments using a cell culture model, and after acute clozapine administration in rat brain. In addition, the expression of the candidate target genes was examined after chronic haloperidol and clozapine treatments in rat brain.
2 REVIEW OF THE LITERATURE

2.1 DOPAMINERGIC SYSTEM IN BRAIN

Dopamine, together with noradrenaline and adrenaline, is a catecholamine neurotransmitter. Dopaminergic neuron cell bodies are located in three major separate nuclei in the midbrain from which they project via nigrostriatal, mesolimbic, and mesocortical systems to various brain regions (Figure 1). Dopamine is also used as a neurotransmitter in the tuberoinfundibular system and in retinal cells. Dopaminergic neurons of the nigrostriatal system are located in substantia nigra pars compacta and innervate the caudate and putamen (together known as the striatum). The nigrostriatal pathway has a fundamental role in the control of movement. Mesolimbic and mesocortical systems originate from the ventral tegmental area. The mesolimbic system is involved in motivated behavior and it projects to the ventral striatum, septum, nucleus accumbens, amygdaloid complex, piriform cortex, and to olfactory tubercle. Blockade of the postsynaptic dopamine receptors in the mesolimbic pathway is hypothesized to result in reduction of positive symptoms of schizophrenia. The mesocortical system implicated in learning and memory ascends its dopaminergic projections to the limbic cortical regions, such as the prefrontal cortex (PFC), and cingulate and entorhinal cortices. The PFC is especially highly innervated by dopaminergic neuron terminals where dopamine is thought to modulate the input for the storage of working memory. Underactivity of this pathway has been hypothesized to result in hypofrontality, a reduced activational status of the frontal cortical regions, which may be related to the negative symptoms of schizophrenia (Stahl, 1996a; Vallone et al., 2000).

Dopamine binds to five distinct G-protein coupled dopamine receptors that have been classified into D₁ and D₂ type dopamine receptors based on their pharmacological, biochemical, and physiological functions. The expression of D₁ and D₂ type receptors is highly overlapping. D₁ type receptors (D₁ and D₃) are coupled to adenylate cyclase (AC) with stimulatory Gₐ-type G-proteins. Binding of an agonist thus induces formation of adenosine 3',5'-cyclic monophosphate (cAMP). The D₁ receptor is the most widespread and expressed at higher levels than other dopamine receptors. D₁ mRNA has been detected in high amounts in the limbic cortical structures, and in the striatum, olfactory tubercle, and nucleus accumbens. The D₃ receptor mRNA is expressed at low levels in comparison to D₁ mRNA, and is detectable in the hippocampus, hypothalamus, and in the cortex. D₁ and D₃ proteins co-localize, for example, in the PFC, anterior cingulate and entorhinal cortices, and in the
hippocampus. D₂ type receptors (D₂ short and long form splice variants, D₃, and D₄) are linked to AC via inhibitory Gᵢ/Gₒ type G-proteins and thus agonist binding decreases cAMP production. In addition, activation of D₂ receptors may cause cell hyperpolarization due to inhibition of voltage-activated Ca²⁺ channels, and by activation of inwardly-rectifying K⁺ channels. The D₂ receptor is expressed by pre- and postsynaptic neurons and serves as the main dopamine autoreceptor. The highest D₂ receptor expression levels have been observed in the striatum, olfactory tubercle, and in the core of the nucleus accumbens. It is also expressed in the substantia nigra and in the ventral tegmental area by dopaminergic projection neurons. Cortical D₂ expression has been detected in the PFC, cingulate, temporal, and entorhinal cortices, and in the hippocampus. Expression of D₃ receptors appears restricted to limbic structures such as the shell of the nucleus accumbens. They are also expressed in the hippocampus, septum, and in the temporal cortex. The D₄ receptors appear highly expressed in the frontal cortex, amygdala, hippocampus, hypothalamus, and mesencephalon (Missale et al., 1998; Nestler et al., 2001a).

![Diagram](image)

**Figure 1.** The major dopaminergic projections in rat brain. Symbols: 1., nigrostriatal, 2., mesolimbic, and 3. mesocortical dopaminergic systems. Abbreviations: Substantia nigra, pars compacta (SN), ventral tegmental area (VTA), caudate putamen (CPU), nucleus accumbens (Acb), prefrontal cortex (PFC), anterior cingulate cortex (Cg cx), and frontal cortex (Fr cx).

### 2.2 GLUTAMATERGIC SYSTEM IN BRAIN

Glutamate is the major excitatory neurotransmitter in the brain. Glutamate is synthesized in the brain from glucose and, in addition to being an amino acid neurotransmitter, it serves as precursor for the inhibitory neurotransmitter gamma-amino-
butyric-acid (GABA). Glutamate is released from the presynaptic terminal by a Ca\(^{2+}\) dependent manner. Synaptically released glutamate binds to two families of glutamate receptors: metabotropic and ionotropic receptors. Metabotropic glutamate receptors belong to the super family of G-protein coupled receptors and are classified into three groups (I, II and III) based on their amino acid sequence homology, signal transduction mechanisms and agonist pharmacology. Activation of metabotropic glutamate receptors is slower than that of ionotropic receptors and they mediate the slow actions of glutamate induced neurotransmission. Metabotropic glutamate receptors are located both in the pre- and postsynaptic neurons. Postsynaptic receptors modulate the actions of glutamate on various ligand and voltage gated ion channels. Binding of glutamate into presynaptic metabotropic glutamate receptors inhibits the presynaptic excitatory glutamatergic and inhibitory GABAergic neurotransmission. Ionotropic glutamate receptors include N-methyl-D-aspartate (NMDA), \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate class receptors named by their selective agonists. The ion channel and glutamate binding site of ionotropic glutamate receptors are situated in the same complex. Ionotropic receptors mediate fast excitatory transmission by agonist binding. Activation of AMPA and kainate receptors induces mainly Na\(^{+}\) and K\(^{-}\) currents and mediates fast synaptic current with a rapid decay. Specific antagonists of AMPA receptors have been identified whereas blockers of kainate receptors are yet to be clearly demonstrated (Nestler et al., 2001c).

The NMDA receptor has several modulatory binding sites (Nestler et al., 2001b). The binding sites include sites for glutamate/aspartate/NMDA, polyamines, glycine, Zn\(^{2+}\), Mg\(^{2+}\), and phencyclidine (PCP). Under the normal resting potential, the ion channel of the NMDA receptor complex is blocked by Mg\(^{2+}\). Depolarization induced by glutamate activation of AMPA and kainite class receptors releases the Mg\(^{2+}\) blockade and thus enables cation flux (Ca\(^{2+}\), Na\(^{+}\), K\(^{-}\)). NMDA receptor function can be modulated by various receptor antagonists. The competitive NMDA receptor antagonists block the binding of the channel agonist glutamate, or the co-agonist glycine, to the receptor. The non-competitive antagonists act on the modulatory sites of the NMDA receptor and the ion channel without competing with glutamate or glycine binding. PCP and related anesthetic drugs, such as MK-801 and ketamine, are non-competitive antagonists that bind into the PCP binding site within the ion channel when the channel is open, and thereby prevent further ion flux. MK-801 is selective and the most potent non-competitive NMDA-receptor antagonist (Wong et al., 1986b).
2.3 FOS AND JUN FAMILY GENES AND THE AP-1 COMPLEX

2.3.1 INDUCTION AND FUNCTION

Fos and Jun family genes belong to the class of immediate early genes (IEG) that are induced in response to various stimuli rapidly in the absence of de novo protein synthesis. The inducing stimuli can consist of growth factors, sensory stimulation, exogeneous and endogeneous toxins, trauma, and neuronal depolarization. Neuronal stimulus activates second messenger pathways leading to activation of kinases, such as extracellular signal regulated kinase (ERK), c-jun N-terminal kinase (JNK) and cyclic AMP dependent protein kinase (PKA), that translocate into the nucleus to phosphorylate and induce the DNA binding of constitutive transcription factors, such as cAMP responsive element binding protein (CREB) and activating transcription factor (ATF) (Figure 2). Their activation ultimately leads to enhanced transcription of the IEGs. Induction of IEGs can be used as a marker of neuronal activation (Dragunow and Faull, 1989; Herdegen and Leah, 1998; Morgan and Curran, 1991; Sagar et al., 1988).

The most studied IEGs are fos (c-fos, fosB, fra-1, and fra-2) and jun family genes (c-jun, junB, and junD). The protein products of fos and jun family gene transcription factors translocate from cytoplasm into the nucleus where they homo- or hetero-dimerize to form an active transcription factor complex (Figure 2). Heterodimers of Fos and Jun proteins (Fos/Jun), and Jun-protein homodimers (Jun/Jun) form activator protein 1 (AP-1) complexes. AP-1 complexes bind with high affinity to AP-1 DNA consensus sequence sites in regulatory regions of Fos-inducible genes. Binding of the AP-1 complex usually activates the transcriptional activity of the target gene but depending on the types of Fos and Jun proteins in the AP-1 complex, may also repress it. In general, Fos/Jun complexes have higher binding affinity to the AP-1 site than Jun/Jun homodimers have (Herdegen and Leah, 1998).

Genes of fos and jun families, like other IEGs, are characteristically induced and downregulated rapidly. Activated IEG mRNA levels usually decay within one hour, and the amounts of the corresponding proteins peak after one hour and decay within three to four hours. Continuous stimulation does not interfere with the fast kinetics. In addition, some stimuli are able to induce secondary waves of IEG expression, however, even these waves are typically dissipated by 12 – 24 hours (Herdegen and Leah, 1998).
Figure 2. Induction and function of *fos* and *jun* family transcription factors.

Fos family transcription factor proteins have been recently divided into three classes (Nestler et al., 1999). As compared to rapid induction of c-Fos, ‘acute’ Fra’s (e.g., FosB, ΔFosB, Fra-1, Fra-2) are induced somewhat later and their expression lasts longer than that of c-Fos. A longer lifespan is observed with the accumulating ‘chronic’ Fra’s, biochemically modified forms of ΔFosB protein, that are expressed after repeated inducing stimuli (Nestler et al., 1999). Chronic disruptions of the nervous system such as seizure, axotomy, transplantation, and repeated exposure to neuroactive agents, such as cocaine and kainic acid, induce the expression of the chronic Fra’s (Bing et al., 1996; Doucet et al., 1996; Herdegen and Leah, 1998; Hope et al., 1994; Kelz et al., 1999; Morris et al., 2000; Nestler et al., 1999; Nye et al., 1995).
2.4 GENE EXPRESSION ANALYSIS IN THE BRAIN

2.4.1 HIGH-THROUGHPUT METHODS FOR GENE EXPRESSION ANALYSIS

The analysis of the relative quantities of expressed mRNA transcripts and alterations in their expression levels allows one to estimate the physiologic and molecular outcomes in response to various stimuli at the gene level. Traditionally, gene expression has been evaluated using methods that are suitable for the simultaneous analysis of only a limited number of genes, such as RNase protection assays, northern blotting and in situ hybridization. Recent advances in genome sequencing projects have greatly increased the number of interesting candidate genes. Traditional methods, although still valid and necessary, are inappropriate for high-throughput screening of gene expression. Many new methods are now available, and the rapid development of the techniques has inspired comparisons between the available choices (Colantuoni et al., 2000; Green et al., 2001; Ishii et al., 2000). A selection of modern methods for high-throughput gene expression analysis is reviewed below.

2.4.1.1 DNA EXPRESSION ARRAYS

DNA expression arrays consist of a large selection of target gene stretches that can be either spotted cDNA clones from libraries, purified PCR fragments of known or unknown sequences, or specifically designed oligonucleotides attached onto a solid surface in high density. Spotting individual clones from selected cDNA libraries prepared from any tissue of interest onto an array allows discovery of novel genes. Furthermore, expression patterns of unknown genes presented by expressed sequence tags (EST) can be precisely investigated with an expression array. The expression array is hybridized with a labeled cDNA or cRNA probe (radioactive or fluorescent) derived from either total RNA or mRNA of any biological source under desired conditions (Figure 3). Hybridization on the array between complementary DNA-DNA or DNA-RNA sequences allows quantification of hybridization intensities that correspond to the relative abundances of expressed mRNA species.

The first expression arrays were developed on relatively large nylon filters (Zhao et al., 1995). Filter arrays, often referred to as macroarrays, utilize radioactive detection, in which cDNA probes from control and test samples are hybridized on separate arrays and the resulting hybridization patterns are compared (Figure 3A). Filter arrays have since been exploited actively in various applications to identify differentially expressed genes.
Microarrays, expression arrays printed on glass microscope slide surface, have enabled a higher density of target gene sequences in considerably smaller format (Chee et al., 1996; Lockhart et al., 1996; Schena et al., 1995). Entire genomes have been printed onto microarrays (Wodicka et al., 1997). Microarray hybridization is based on simultaneous hybridization of two fluorescently tagged samples that bind to the cognate gene sequences on the array (Figure 3B). Simultaneous probe binding onto a single array slide reduces variation due to variability in arrays such as may occur in serial hybridization using filter arrays.

A.  

B.  

**Figure 3.** Schematic presentation of gene expression profiling using (A) filter array, and (B) microarray.

DNA array technology has been successfully used in various applications such as characterization of signal transduction pathways (Roberts et al., 2000), cancer classification (Alizadeh et al., 2000; Golub et al., 1999; Perou et al., 1999; Ramaswamy et al., 2001; van 't Veer et al., 2002), analysis of changes in gene copy number (Pollack et al., 1999), in detection of modifications in immune response (Huang et al., 2001; Hämäläinen et al., 2001; Irving et al., 2001), and in discovery and analysis of disease-related genes (Heller et al., 1997). Furthermore, large scale profiling of changes in gene expression in response to drug treatment has been successfully applied, and is an important application of the methodology (Braxton and Bedilion, 1998; Debouck and Goodfellow, 1999; Gerhold et al., 2001; Hughes et al., 2000).
DNA expression array analysis has only recently been utilized in neuroscience applications. Research has focused on various subjects, such as to identify genes involved in brain development (Miki et al., 2001; Mody et al., 2001), and in neuronal diseases (Brown et al., 2001; Chabas et al., 2001; Whitney et al., 2001), and to dissect the molecular characteristics of anatomically specific nuclei (Zirlinger et al., 2001). Furthermore, strain-specific expression patterns in mouse brain regions have been identified using DNA array analysis (Sandberg et al., 2000). Expression array technique has identified alterations in gene expression induced by drug treatments also in neuronal tissue (Kittler et al., 2000; Loguinov et al., 2001; Thibault et al., 2000; Yamada et al., 2000). Recently, microarrays have been utilized to study gene expression in the brain of schizophrenic subjects (Hakak et al., 2001; Middleton et al., 2002; Mimmack et al., 2002; Mirmics et al., 2000; Vawter et al., 2001) (see section 2.5.2).

2.4.1.2 DIFFERENTIAL DISPLAY

Differential display is based on polymerase chain reaction (PCR) amplification of mRNA molecules, and on the separation of resulting cDNAs with polyacrylamide gel electrophoresis to identify differentially expressed genes (Liang and Pardee, 1992). Two pools of oligonucleotide primers are used. Oligo(dT) primers with variable two nucleotides in their most 3’ end anchor to the poly A’ tail of mRNAs. The other primer is short and arbitrary in sequence, and anneals at different positions relative to the oligo(dT) primer. mRNA molecules with the two primers are amplified after reverse transcription into cDNA, and resolved on a DNA sequencing gel. cDNA fragments obtained by differential display are strongly dependent on sequence specificity of either primer and less dependent on the prevalence of mRNA species.

Differential display has been widely used and reviewed (~3900 citations in PubMed, NCBI, as of February 2002). Critical evaluation of differential display has revealed benefits and caveats in the methodology (Wan et al., 1996). Differential display is affordable to use and requires small amounts of either total or mRNA. In addition, it identifies both rare and abundant transcripts of upregulated and downregulated mRNA species. The technique can be used to quickly evaluate the proportion of mRNAs with altered expression levels. This allows redesign of the experiment if necessary. However, differential display has been shown to produce ~50% true positive hits and to result in a substantial occurrence of redundant transcripts. Redundancy is due to annealing of several degenerate short primers to distinct
regions of the same mRNA. Alternatively, the same degenerate primer may hybridize to several mRNAs coupled with oligo(dT) primers of various nucleotide variations. The sequences obtained by differential display are short and are amplified from the typically untranslated 3’ end of the transcripts. This leads to difficulties in identification of the transcript. However, sequence comparisons with several EST databases available may help in transcript identification. Differential display in combination with DNA expression arrays represents a potential application of the methodology: candidate genes found by differential display could be spotted onto DNA expression arrays for high-throughput expression analysis. Differential display has been suggested as a potentially applicable method for study of psychiatric illnesses (Flanigan and Leslie, 1997).

2.4.1.3 SERIAL ANALYSIS OF GENE EXPRESSION

Serial analysis of gene expression (SAGE) is an open-system approach, like differential display, to detect differential gene expression (Velculescu et al., 1995). SAGE utilizes short cDNA sequence tags directed to recognize individual cloned mRNA sequences. The tags are produced from the 3’ end of transcripts with a restriction endonuclease reaction that cleaves each transcript in a sequence specific location. Thus, each tag can be identified according to its location. The production of tags is proportional to the abundance of the transcript in the pool of mRNA molecules. Tags are then ligated together to produce long stretches of tags. Tag-polymers are cloned and sequenced in series allowing simultaneous identification of a number of tags. Tag sequences (9 bp in length) are then used to search gene databases to identify the gene or EST.

SAGE enables identification of novel genes, in addition to known genes, and quantification of the gene expression levels by the abundance of the tags. It has been estimated that in a cell line preparation, a collection of ~250,000 tags enables evaluation of transcript concentration of all but the rarest transcripts (Sutcliffe, 2001). The number of tags needed to identify differential gene expression of more complex tissues is likely much higher. Thus, expenses may restrict analysis of differentially expressed genes by SAGE in a complex tissue. Recently, a direct comparison of the accuracy of SAGE and oligonucleotide-based expression array has been performed (Ishii et al., 2000). The results indicate that the methods correlate well in both absolute expression analyses and in comparative analyses in a cell culture model. Overall, the correlation was better for genes with high absolute expression levels, and for genes with greater changes in expression. In neuroscience research, SAGE has
been applied to identify genes with altered expression in an epilepsy model (Hendriksen et al., 2001), to collect differentially expressed transcripts from aneuploid mouse brain (Chраст et al., 2000), and to generate an expression profile of normal rat hippocampus (Datson et al., 2001). Furthermore, SAGE has been recently applied to evaluate alterations in gene expression in bipolar disorder (Sun et al., 2001).

2.4.1.4 OTHER METHODS

Total analysis of gene expression (TOGA) is a PCR-based method for massive extraction of gene expression patterns (Sutcliffe et al., 2000). Similar to differential display and SAGE, TOGA utilizes sequences near the 3' end of the mRNA molecules. In TOGA methodology, 3' sequences adjacent to the poly A<sup>+</sup> tail are excised by restriction endonuclease and the resulting pieces are coupled to primers that are used for PCR amplification. During PCR, high fidelity base pairing is achieved by alternating the nucleotide combination of four-nucleotide long primers while a fluorescent label is inserted. The resulting nonoverlapping pools of PCR templates are resolved by automated gel electrophoresis and the results are collected into a database. TOGA has not been widely applied, but has been employed in neuropsychopharmacology to search for gene expression profiles in response to long-term treatment with clozapine in mouse striatum (Thomas et al., 2001a) (see section 2.6.3.2).

Gene expression analysis using subtraction hybridization is a traditional technique to collect a number of differentially expressed genes (Jiang et al., 1995). Subtraction hybridization is based on hybridization of adaptor-ligated first-strand cDNAs of tester mRNA with an excess of driver mRNA. The remaining unhybridized single-stranded cDNAs are collected and used as templates for second-strand cDNA synthesis by PCR amplification. The method has been further developed by using cDNA plasmids in phage vectors as both drivers and testers (Rubenstein et al., 1990). Subtractive hybridization is best suited for identification of transcripts with pronounced alterations in expression. In addition, it detects only upregulated genes in the tester RNA population. In neuroscience research, subtractive cloning has been applied for example to detect differences in brains of patients with Down's syndrome (Labudova et al., 1999), and to identify candidate genes for autism (Colantuoni et al., 2000).

Representational difference analysis (RDA) is also a PCR-based method that was originally directed to genomic analysis (Lisitsyn and Wigler, 1993), but has been modified for
analysis of cDNA (RDA for cDNA) for detection of differentially expressed genes (Hubank and Schatz, 1994). Identification of differentially expressed genes is based on analysis of restriction endonuclease fragments that are present in one population but not in another. RDA for cDNA is capable of detecting rare transcripts but if not properly performed it is prone to false positives and negatives (Green et al., 2001). RDA for cDNA has been applied in neuroscience research to study, for example, gene expression patterns of central nervous system progenitor cells (Geschwind et al., 2001), to identify transcripts in cortical ischaemia (Bates et al., 2001), and to analyze viral candidate cDNAs in Creutzfeldt-Jakob disease (Dron and Manuelidis, 1996).

Tissue microarray technology represents another aspect of high-throughput expression analysis (Kononen et al., 1998). Tissue microarray methods provide the expression patterns of any gene of interest simultaneously in thousands of tissue specimens in a parallel fashion. Thus, tissue microarrays represent a different aspect of expression profiling. The technology can be applied to detect tissue morphology, mRNA or protein expression, or to detect DNA. Tissue microarrays have been used with notable success in cancer research (Kallioniemi et al., 2001).

2.5 SCHIZOPHRENIA

Schizophrenia is a chronic brain disorder that affects approximately 1% of the population worldwide (Jablensky et al., 1992). Development of the clinical syndrome recognized as schizophrenia is slow and is usually identified during the second or third decade of life. There is a strong genetic component in the vulnerability of schizophrenia (Gottesman and Erlenmeyer-Kimling, 2001). In addition to sporadic cases, familial forms of schizophrenia have been documented with heritability of as high as 80% in twin studies (Cannon et al., 1998). Several regions in many chromosomes have been linked to the disease (Baron, 2001). Various hypothesis of the origin of schizophrenia have been postulated, but currently there is no consensus regarding the mechanisms involved. Nonetheless, the pathogenesis appears to involve neurochemical and neuropathological abnormalities (Lewis and Lieberman, 2000).
2.5.1 BIOLOGY OF SCHIZOPHRENIA

Etiological hypotheses of schizophrenia include defective fine-tuning of brain development (such as neurite formation, synaptogenesis, neuronal pruning and apoptosis), malfunctioning neural connectivity within distributed neural circuits and neurotransmitter systems, multiple hit hypothesis of genetic and non-genetic factors, and misregulation of information processing (Andreasen, 2000). Heterogenic symptoms of schizophrenia include negative and positive symptoms, and cognitive deficits that are not necessarily manifested at the same time in a patient, and the diversity of their appearance differs among affected individuals. Classical negative symptoms can be characterized as defective mental functions, such as attentional impairment and affective blunting, whereas positive symptoms include extra sensations such as hallucinations and delusions. Positive symptoms are generally well treated with antipsychotic drug medication, whereas negative symptoms respond poorly to medication, especially in the chronic phase of the disease.

Schizophrenia appears as a generalized disease of the cortex as indicated by functional and morphological neuronal dysregulation. Magnetic resonance imaging (MRI) has revealed several brain regions as altered in schizophrenia (Shenton et al., 2001), however, the most consistent finding is an increase in ventricular space accompanied with a slightly reduced brain tissue volume. The brain regions solidly implicated as reduced using MRI include the temporal and frontal lobes and thalamus. Reduced brain volume is not associated with gliosis, which suggests that schizophrenia is not a neurodegenerative disease, however, such possibility for the etiology of the disease should not be excluded (Lieberman, 1999). Cortical regions implicated in the limbic circuitry seem affected in schizophrenia (Harrison, 1999a). The limbic cortex (limbic association cortex), a ring of phylogenetically primitive cortical substratum surrounding the brain stem, is a functionally inter-related cortical entity which plays an important role in learning, memory, and emotions (Papez, 1937). The limbic cortex is located in the medial and ventral surfaces of the frontal lobe, the medial surface of the parietal lobe, and the anterior tip of the temporal lobe, and includes the cingulate, prefrontal and association cortices, and the parahippocampal area (Kandel and Kupfermann, 1995; Kupfermann, 1995). The first evidence for abnormal cortical function in schizophrenia by Weinberger and colleagues (Weinberger et al., 1986) reported decreased cerebral blood flow in the PFC. Functionally, schizophrenic subjects often display impaired cognitive processes, one of the inclusion criteria of the disease, that involve the neuronal circuitries of the PFC (Goldberg and Weinberger, 1988). Activation of the limbic cortex also appears
abnormal (Carter et al., 1997; Haznedar et al., 1997; Nordahl et al., 2001) especially during psychotic symptoms (Sabri et al., 1997; Silbersweig et al., 1995). Aberrant neuronal findings in the PFC include reduced gray matter volume (Buchanan et al., 1998; Schlaepfer et al., 1994), which may result at least partly from increased neuronal packing density (Selemmon et al., 1995; Selemmon et al., 1998), reduced size of various neuronal populations (Pierri et al., 2001; Rajkowska et al., 1998), from selective reduction in the dendritic complexity (Kalus et al., 2000), and from reduced presynaptic terminals (Glantz and Lewis, 1997). Decreased GABA mediated neurotransmission has been reported in several regions of the limbic cortex, including the prefrontal and anterior cingulate cortices (Akbarian et al., 1995; Benes and Bird, 1987; Benes et al., 1991; Benes et al., 1992; Benes et al., 1996; Benes et al., 2001; Pierri et al., 1999; Volk et al., 2000), although reports on anterior cingulate cortex have been somewhat conflicting (Akbarian et al., 1995; Arnold et al., 1995; Pakkenberg, 1993; Selemmon et al., 1998). Entorhinal-hippocampal region also appears affected (Arnold et al., 1991a; Arnold et al., 1991b; Cotter et al., 1997; Harrison, 1999a; Jakob and Beekmann, 1986; Jakob and Beekmann, 1989), however, entorhinal abnormalities remain unclear (Akil and Lewis, 1997; Krimer et al., 1997).

2.5.1.1 DOPAMINE AND SCHIZOPHRENIA

There is both indirect and direct evidence suggesting that abnormal dopaminergic neurotransmission is intimately involved in the etiology of schizophrenia. The major indirect indicators are the capability of all clinically beneficial antipsychotic drugs to antagonize dopamine D2 receptors (Creese et al., 1976; Seeman et al., 1976). In addition, long-term administration of dopamine-releasing drugs, such as amphetamine, methamphetamine, and cocaine are able to induce paranoia-like schizophrenic symptoms in healthy humans, and to aggravate the symptoms of psychotic patients. Direct evidence supporting abnormal dopaminergic functioning in schizophrenia has been provided by imaging studies. Presynaptic action of dopamine has been reported increased in the striatum of schizophrenic patients (Hietala et al., 1995). Striatal release of dopamine by administration of amphetamine appears abnormally enhanced as compared to normal controls, which suggests larger pools of releasable dopamine in schizophrenia (Abi-Dargham et al., 1998; Breier et al., 1997; Laruelle et al., 1996). In addition, striatal dopamine receptors appear to be altered in schizophrenia. An early study demonstrated an increased density of striatal D2 receptors in schizophrenia (Wong et al., 1986a), but studies with more specific D2 receptor ligands have not been able to repeat
the result (Farde et al., 1990; Hietala et al., 1994). It appears that there is heterogeneity regarding D₂ receptor amounts among schizophrenics and a group of patients with increased D₂ receptor density may exist (Hietala et al., 1994). A recent study supports altered D₂ receptor functioning in schizophrenia since increased baseline occupancy of striatal D₂ receptors both in drug-naïve and medicated schizophrenic patients has been demonstrated (Abi-Dargham et al., 2000). Other studies have also revealed abnormalities in D₂ receptor amounts in the temporal and anterior cingulate cortices in schizophrenia (Goldsmith et al., 1997; Suhara et al., 2002).

The dopamine hypothesis of schizophrenia has been criticized due to its inability to address some aspects. Although the majority of antipsychotic drugs occupy a considerable proportion of D₂ receptors in clinically effective doses, there are effective antipsychotic drugs, such as clozapine, which are considerably less potent D₂ receptor antagonists. Furthermore, D₂ receptor antagonism is immediate whereas clinical antipsychotic effect develops very slowly. Hence, other models to explain symptoms and occurrence of schizophrenia have been explored.

2.5.1.2 GLUTAMATE AND SCHIZOPHRENIA

Administration of low doses of the non-competitive NMDA receptor antagonists MK-801, PCP, or ketamine in healthy humans produces psychotropic effects that resemble those of schizophrenia (Javitt and Zukin, 1991; Krystal et al., 1994; Malhotra et al., 1996). In schizophrenic patients, their administration aggravates the appearance of psychotic symptoms, especially positive symptoms of schizophrenia (Lahti et al., 1995a; Lahti et al., 1995b; Malhotra et al., 1997). Studies using regional cerebral blood flow and glucose utilization as an indicator of brain metabolic activity have reported that MK-801 and ketamine activate neurons in the limbic system, such as in the anterior and posterior cingulate cortices, entorhinal cortex, and in the anterior thalamic nucleus (Duncan et al., 1998a; Lahti et al., 1995b; Nehls et al., 1988; Nehls et al., 1990). Interestingly, antipsychotic drug treatment partially reduces psychotic symptoms elicited by non-competitive NMDA-receptor antagonists (Lahti et al., 1995a; Malhotra et al., 1997), and blocks alterations in brain metabolism in experimental animals (Duncan et al., 1998b; Duncan et al., 2000). This evidence has led to a hypoglutamatergic model of schizophrenia, which postulates inadequate glutamatergic neurotransmission as a causative factor in the disease (Goff and Coyle, 2001). Recently, schizophrenia-type behavior that was reversible by antipsychotic drugs has been
reported in mice with reduced NMDA receptor expression (Mohn et al., 1999), which further supports glutamatergic abnormalities as potentially predisposing to schizophrenia. Dysfunction of glutamatergic system in schizophrenia is not in conflict with the dopamine hypothesis since reciprocal connections within the glutamatergic and dopaminergic systems are well established in the forebrain. Projection neurons from the cortex, such as the PFC, use glutamate as their neurotransmitter to innervate striatum. Furthermore, striatal dopaminergic projection neurons innervate the PFC through the mesocorticolimbic dopaminergic system.

Brain regions affected by non-competitive NMDA receptor antagonists have been studied in more detail using expression of IEGs as a marker of neuronal activation. An early report by Dragunow and Faul implicated several limbic cortical regions and some midbrain structures, since these regions express c-Fos protein in response to acute MK-801 treatment (Dragunow and Faul, 1990). c-Fos induction in the cingulate, retrosplenial, and entorhinal cortices has been later verified in several studies (Gass et al., 1993; Hughes et al., 1993; Nääkö et al., 1996b; O’Neill et al., 1998; Väisänen et al., 1999). Furthermore, MK-801 administration induces expression of heat shock proteins -70 and -72 in the posterior cingulate and retrosplenial cortices (Sharp et al., 1991a). Induction of heat shock proteins can be attenuated by administration of antipsychotic drugs (Nääkö et al., 1996a; O’Neill et al., 1998), and by fluoxetine, a selective serotonin reuptake inhibitor (Tomitaka et al., 2000). In addition, MK-801 treatment increases the expression of brain derived neurotrophic factor (BDNF) mRNA in the cingulate and entorhinal cortices (Castrén et al., 1993; Hughes et al., 1993; Lindén et al., 2000), and induces the expression of cAMP regulatable transcription factors in several cortical regions, including the cingulate cortex (Storvik et al., 2000). In the entorhinal cortex, the increase of BDNF is prevented by haloperidol and clozapine treatments (Lindén et al., 2000). Expression of IEGs and BDNF is likely related to MK-801 induced neuronal injury in these brain regions. MK-801 induces reversible and age-dependent neuronal vacuolization in the posterior cingulate and retrosplenial cortices (Farber et al., 1993; Fix et al., 1993; Olney et al., 1989), which can be prevented by antipsychotic drugs (Farber et al., 1993), certain anticholinergic drugs and GABAergic agents (Olney et al., 1991), as well as by α2-adrenergic agonists (Farber et al., 1995). A recent report suggests that neuronal damage induced by MK-801 treatment is caused by a blockade of NMDA receptors in the reticular nucleus of thalamus, from which neuronal projections ascend to the limbic cortex (Sharp et al., 2001).
2.5.2 GENE EXPRESSION IN SCHIZOPHRENIA

Gene expression profiling has been recently applied to search for genes involved in schizophrenia. Due to inherent variation in the etiology of schizophrenia, the characterization of single genes related to the disorder is problematic. However, a few clinical expression array studies have successfully implicated altered expression of genes in various pathways in schizophrenia. The first report of schizophrenia-specific gene expression by Mirnics et al. focused on gene expression in the PFC of medicated patients (Mirnics et al., 2000) and identified decreased expression of functionally related pathway-specific gene groups of presynaptic secretory machinery, GABAergic neurotransmission, glutamate mediated signaling, energy metabolism, growth factors, and receptors. Independent methods confirmed the reduction in presynaptic genes N-ethylmaleimide sensitive factor, synapsin II, synaptotagmin 1, and synaptotagmin V mRNA expression in several schizophrenic subjects. In addition, decreased expression of regulator of G-protein signaling 4, a postsynaptic protein, was observed (Mirnics et al., 2001a). Recently, the same group further shed light on the reduced expression of genes involved in metabolism in the PFC (Middleton et al., 2002). Genes involved in ornithine and polyamine metabolism, mitochondrial malate shuttle system, transcarboxylic acid cycle, aspartate and alanine metabolism, and ubiquitin metabolism appeared decreased by data clustering, and a reduced expression of the mRNAs for cytosolic malate dehydrogenase type 1, mitochondrial glutamate-oxaloacetate transaminase type 2, ornithine decarboxylase antizyme inhibitor, and ornithine aminotransferase was confirmed. As the majority of neuronal metabolism occurs at synaptic sites the data supports their previous findings on reduced synaptic transmission. In monkey brain, all but one of the genes implicated as decreased in schizophrenia by Mirnics and co-workers were not affected by chronic haloperidol treatment (Middleton et al., 2002; Mirnics et al., 2000; Mirnics et al., 2001a), suggesting that the gene expression changes in schizophrenics were in general not due to medication with haloperidol. There are supporting data indicating decreased expression of presynaptic markers in the PFC (Glantz and Lewis, 1997), anterior cingulate cortex (Eastwood and Harrison, 2001), hippocampus (Webster et al., 2001), and cerebellum (Eastwood et al., 2001) in schizophrenia suggesting a synaptic pathology. Supporting data for altered GABAergic neurotransmission also exists; the PFC of schizophrenic patients reportedly have decreased expression of GABA membrane transporter (Pierri et al., 1999) and of glutamic acid decarboxylase (GAD) 67, an enzyme involved in GABA synthesis (Akbarian et al., 1995; Volk et al., 2000).
Furthermore, a reduced expression of several genes involved in neuronal myelination, especially in oligodendrocyte function, has been observed in the PFC of elderly medicated schizophrenics (Hakak et al., 2001). Expression of gene groups for GABA neurotransmission, signal transduction, receptors, ion channels, and transporters was predominantly increased in schizophrenic subjects in comparison to controls. Potentially abnormal neuronal myelination is further supported by a recent report which stated an increased expression of apolipoproteins 1, 2 and 6 in schizophrenia using DNA expression arrays and quantitative PCR (Mimmack et al., 2002). In addition, alterations in the expression of genes related to proteolysis, signal transduction, transcription, and synaptic function have been reported in the PFC, middle temporal gyrus, and cerebellum in a preliminary microarray study of schizophrenia (Vawter et al., 2001).

It appears that schizophrenia induces a different set of expressed genes from those in control subjects, and the studies indicate that a variety of genes in many neuronal pathways and processes may be involved. The discrepancies in the results obtained in the studies may be at least partly explained by different demographics of study subjects, variable post-mortem intervals, differences in the DNA expression arrays used, and by the confounding effects of long-term antipsychotic drug treatments. Nevertheless, expression profiling provides substantial potential to further the understanding of the etiology of schizophrenia.

2.6 ANTIPSYCHOTIC DRUGS

Antipsychotic drug treatment is among the principle means of treatment for schizophrenia. In general, 5-10% of schizophrenic patients recover fully. Approximately 30% of schizophrenics have adequate but partial response to antipsychotic drug medication, and another 30% have inadequate but partial response. About 20-25% of schizophrenics do not benefit from any classical antipsychotic drug treatment (Tammenga, 1999). Antipsychotic drugs have been classified into typical (classical) and atypical (novel) antipsychotics based on their affinities to dopamine D1 and D2 receptors and to the serotonin receptor 5-HT2 (Meltzer et al., 1989). In addition, therapeutic efficacy as well as propensity to induce extrapyramidal side effects discriminates typical and atypical antipsychotic drugs. Although antipsychotic drugs bind receptors immediately after their administration, the clinical antipsychotic effect develops slowly over the first weeks of treatment. The mechanisms behind the delayed drug effectiveness are unknown.
2.6.1 TYPICAL ANTIPSYCHOTIC DRUGS

The first typical antipsychotic drug identified was chlorpromazine, which was originally tested as an antihistamine agent in the 1950's. Chlorpromazine has affinity to D₁ and D₂ receptors, as well as to several other neurotransmitter receptors. Concomitant with the discovery of chlorpromazine, a number of other antipsychotic agents that bind to D₂ receptor were developed. The antipsychotic efficacy of typical antipsychotic drugs has been shown to correlate with dopamine receptor occupancy. Haloperidol is the most recognized typical antipsychotic agent that is highly effective at relatively low doses. It is beneficial in the treatment of the positive symptoms of schizophrenia with little effect on the negative symptoms. Haloperidol is the prototype among typical antipsychotic agents since it is a high affinity D₂ receptor antagonist (D₂, D₃, and D₄). In addition, it has measurable affinity for the sigma binding site, for 5-HT₂A serotonin, and for α₁ noradrenergic receptors. In clinically effective doses, haloperidol dose dependently blocks 65-85% of striatal D₂ receptors, and extrapyramidal symptoms appear as D₂ binding increases to 80% (Farde et al., 1992; Nordstrom et al., 1995). The effects of haloperidol on extrastriatal regions have been less well characterized. However, D₂/D₃ receptor binding of typical antipsychotic drugs has been detected in the thalamus and temporal cortex (Bigliani et al., 1999; Farde et al., 1997), and a recent report showed that haloperidol binds efficiently both in striatal and cortical D₂ receptors (Talvik et al., 2001). In addition, haloperidol treatment has been reported to induce dopamine release mainly in the striatum, and also in the PFC (Moghaddam and Bunney, 1990), and to activate neurons in the PFC (Kim et al., 2001b).

Typical antipsychotic drugs have a propensity to cause various side effects. In long-term use high striatal D₂ occupancy by haloperidol, as well as by other typical antipsychotic drugs, may lead to the appearance of extrapyramidal symptoms, such as parkinsonism and tardive dyskinesia. Other side effects of typical antipsychotic drugs are related to their affinities to various neurotransmitter receptors. For example, sedation is caused by blockade of H₁ histamine receptors, hypotension by antagonism of α₁ receptors, and dry mouth, constipation, and blurred vision result from the blockade of muscarinic cholinergic receptors (Nestler et al., 2001d; Stahl, 1996b; Tamminga, 1999).
2.6.2 ATYPICAL ANTPSYCHOTIC DRUGS

The side effects and other unwanted properties of typical antipsychotic agents have prompted a search for better tolerated and more effective antipsychotic drugs. The new generation of antipsychotics, so-called atypical antipsychotics, are exemplified by clozapine. In contrast to typical antipsychotics, clozapine has relatively low affinity to D₂ receptors. Therapeutic doses of clozapine block only 30-60% of striatal D₂ receptors and have a lower propensity to induce extrapyramidal symptoms (Farde et al., 1992; Nordstrom et al., 1995). Moreover, clozapine is effective towards the negative symptoms of schizophrenia, and in the treatment of patients that do not respond well to typical antipsychotic drugs (Kane et al., 1988). Clozapine binds to D₁ dopaminergic, 5-HT₂ serotonergic receptors, D₂ receptors, muscarinic cholinergic receptors, H₁ histaminergic, and to α₁ and α₂ adrenergic receptors (Kinon and Lieberman, 1996). It is not clear, which of the pharmacologic properties of clozapine are crucial for its superior effectiveness profile. Although clozapine does not specifically target cortical D₂ receptors (Talvik et al., 2001), it appears to increase dopamine release especially in the PFC (Moghaddam and Bunney, 1990). In addition, it induces dopamine release in the striatum and nucleus accumbens, but the effect appears less profound than in the PFC (Moghaddam and Bunney, 1990). Despite the tremendous improvement in the treatment of schizophrenia by clozapine administration, it may cause a serious side effect in approximately 1% of patients; agranulocytosis, a decreased production of white blood cells, is a potentially fatal condition accompanied with clozapine treatment. For this reason, clozapine treatment is accompanied by close monitoring of patient blood cell counts. Other atypical antipsychotics include olanzapine, risperidone, and quetiapine. The clinical efficacy of olanzapine appears similar to clozapine, whereas risperidone and quetiapine seem less effective than clozapine. Also other atypical antipsychotic agents appear to share the preference of cortical action (Bigliani et al., 2000; Stephenson et al., 2000).

Various aspects of the cellular and molecular effects produced by antipsychotic drugs have been investigated in vitro using neuronal cell lines or primary neuronal cultures as a model system. Several studies have employed monoaminergic PC12 cell lines that are derived from rat pheochromocytoma and can be differentiated into neuronal-like cells with nerve growth factor (Greene and Tischler, 1976). PC12 cells display functional D₂ receptors that are sensitive to specific receptor agonists and antagonists (Courtney et al., 1991), as well as receptors for noradrenaline (Schomig et al., 1988). Both typical and atypical antipsychotic drugs have been shown to inhibit glucose transport in PC12 cells, which may have direct
implications for imaging studies in medicated schizophrenic patients (Ardizzone et al., 2001; Dwyer et al., 1999). Antipsychotic drugs appear to have a wide range of effects on ion transport in vitro. Ca^{2+} has been shown to be crucial for actions elicited by haloperidol (Esteve et al., 1995) and chlorpromazine (Choi et al., 2001) in PC12 cells. Typical antipsychotics have been shown to block low-voltage activated L-type Ca^{2+} channels (Fletcher et al., 1994; Ito et al., 1996; Lee et al., 1999), high-voltage-activated T-type Ca^{2+} channels (Santi et al., 2002), and voltage-activated K+ current in vitro (Nakazawa et al., 1995). Furthermore, in vitro studies have revealed that both haloperidol and clozapine modulate NMDA receptor function (Leveque et al., 2000). Haloperidol has been shown to interact with the glycine binding site in the NMDA receptor complex (Fletcher and MacDonald, 1993). These examples of the fundamental effects that antipsychotic drugs exert in vitro have encouraged the use of cultured cells to model the neuroactive functions of antipsychotic drug treatments.

2.6.3 GENE EXPRESSION BY ANTIPSYCHOTIC DRUGS

Antipsychotic drug treatments have various effects on gene expression in vivo. Gene expression altered by the prototype antipsychotic drugs haloperidol and clozapine has been used to evaluate the ability of antipsychotic drug treatments to modulate transcription. The majority of the data has been gathered from experimental animals but clinical studies, especially on the long-term drug treatment effects exist. In general, typical and atypical antipsychotic drugs, as well as acute and chronic treatments, appear to have different effects on gene expression. Alterations in gene expression by acute and chronic drug administration are reviewed below regarding fos and jun family immediate early genes, and a number of other genes including some neurotransmitter receptors.

2.6.3.1 FOS AND JUN FAMILY GENE EXPRESSION

Antipsychotic drugs induce the expression of c-fos mRNA and protein in various brain regions depending on the drug type and the duration of the treatment. Acute effects of haloperidol and clozapine treatments on c-fos expression in rodents have been studied in great detail. The induction patterns of c-fos mRNA or protein in the rat brain after acute and chronic haloperidol and clozapine treatments are summarized in Table 1. The expression of c-fos in these studies has been investigated immediately after (1-4 hours) a single (acute)
injection or after the last of the chronic injections. Briefly, acute haloperidol administration induces c-Fos in several brain regions, and the effect is the most pronounced in the dorsolateral striatum. Acute clozapine induces c-Fos especially in the PFC. After chronic treatments, the effects are predominantly detected in the same brain regions as acute effects, but the induction is often attenuated from the acute response. The chronic actions of haloperidol and clozapine on fos and jun family gene expression are significantly less studied than the acute effects despite the need for long-term antipsychotic drug administration in treatment of schizophrenia.

Table 1. Effects of acute and chronic treatments with haloperidol or clozapine on c-Fos mRNA or protein induction.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Haloperidol</th>
<th>Clozapine</th>
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<td>Acute</td>
<td>Chronic</td>
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<tr>
<td>Ant Cg</td>
<td>↑ [5]</td>
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<td>Entorhinal ex</td>
<td>↑↑ [1, 5]</td>
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<tr>
<td>AcbC</td>
<td>↑↑ [1, 5]</td>
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<tr>
<td>AcbSh</td>
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<td>DLS</td>
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<td>Amygdala</td>
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<td>Septum</td>
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Abbreviations: PFC, prefrontal cortex; Ant Cg, anterior cingulate cortex; AcbC, nucleus accumbens, core; AcbSh, nucleus accumbens, shell; DLS, dorsolateral striatum.


The expression patterns of fos and jun family genes other than c-Fos have not been thoroughly studied after antipsychotic drug treatments. Expression of ΔFosB has been reported to increase by chronic antipsychotic drug treatment in rat brain. Long-term haloperidol treatment induces ΔFosB immunoreactivity in the striatum, nucleus accumbens and in the PFC (Atkins et al., 1999; Hiroi and Graybiel, 1996; Vahid-Ansari et al., 1996). The effects of chronic clozapine treatment on ΔFosB induction are less consistent. Clozapine has been reported to induce ΔFosB in the medial layers of frontal cortex (Rodriguez et al., 2001),
but to have no effect in the PFC (Atkins et al., 1999). The atypical antipsychotic drugs risperidone and olanzapine appear to induce ΔFosB in the striatum, and the induction is hypothesized to result from the D₂ binding properties of these drugs (Atkins et al., 1999).

The components of the AP-1 complex can be modulated by dopamine receptor antagonists. Acute administration of haloperidol and the D₂ antagonist (--)-sulpiride induces the expression of c-Fos, FosB, Fra-1, c-Jun and JunD proteins, whereas treatment with acute SCH23390, a D₁ selective antagonist, results in upregulation of FosB, Fra-1, and JunD proteins (Ozaki et al., 1997; Ozaki et al., 1998). Acute and chronic haloperidol treatments increase the DNA-binding activity of the AP-1 complex in the rodent striatum and whole brain extracts (Atkins et al., 1999; Nguyen et al., 1992; Ozaki et al., 1997; Ozaki et al., 1998), while the effects of clozapine on AP-1 complex binding activity remain to be elucidated.

2.6.3.2 EXPRESSION OF NEUROTRANSMITTER RECEPTORS AND OTHER GENES

Neurotransmitter systems are widely regulated by antipsychotic drugs in several brain regions after acute but most notably after chronic administrations. The vast majority of studies have been performed using receptor specific ligand binding assays which measure the number of receptors. In addition, studies on mRNA regulation have been performed for some neurotransmitter receptors. Dopamine receptors, which appear as potentially involved in the etiology of schizophrenia, are classically regulated by antipsychotic treatments. Characteristically, striatal D₂-like receptor binding is significantly increased in rodents by typical but not by atypical antipsychotic drugs (Florijn et al., 1997; Joyce, 2001; Rupniak et al., 1985; See et al., 1990; Tarazi et al., 1997). D₂ receptor upregulation occurs also in humans (Silvestri et al., 2000). Striatal modulation by chronic treatment with typical antipsychotic drugs, however, appears to be more related to extrapyramidal symptoms than to antipsychotic efficacy. Prolonged administration of haloperidol and clozapine has been reported to induce D₂ receptors also in the cortex, including the prefrontal and parietal cortices (Florijn et al., 1997; Janowsky et al., 1992; Lidow and Goldman-Rakic, 1994; Lidow et al., 1998). Atypical antipsychotic drugs olanzapine, risperidone and quetiapine also increase D₂ receptors in the PFC but also in the striatum, which is potentially related to their ability to induce some extrapyramidal side effects (Tarazi et al., 2001). Receptor binding of cortical D₁ receptors following long-term antipsychotic drug treatments appears either not affected or suppressed (Florijn et al., 1997; Lidow et al., 1998; Tarazi et al., 1997). Studies on the expression levels of dopamine receptor mRNAs by haloperidol have been controversial; drug treatment has
been reported either to have no effect on dopamine receptor mRNA expression in the striatum or to upregulate it (Damask et al., 1996; Fox et al., 1994; Hurley et al., 1996; Srivastava et al., 1990). Expression of D3 receptor mRNA does not appear regulated by chronic haloperidol (Joyce, 2001) whereas clozapine has been reported to induce it (Hurley et al., 1996). Thus, although dopamine receptor binding increases by antipsychotic drug treatments, the regulation on the mRNA level remains somewhat controversial.

Regulation of serotonin receptors by antipsychotic drug treatments has been studied in part due to significant binding of clozapine to this class of receptors. Acute clozapine and haloperidol administrations in rat transiently increase the expression of 5-HT2A receptor (Buckland et al., 1997; Burnet et al., 1996). In long-term administration, however, clozapine decreases 5-HT1C, 5-HT1A, 5-HT2A, -HT3C, and 5-HT6 receptors (Ase et al., 1999; Buckland et al., 1997; Burnet et al., 1996; Frederick and Meador-Woodruff, 1999; Hietala et al., 1992; Kuoppamaki et al., 1993; Kuoppamaki et al., 1995). Haloperidol in general does not appear to affect the expression of serotonin receptors, but has been reported to have region-specific effects on 5-HT1A receptor mRNA expression (Ase et al., 1999).

The hypothesized role of glutamate in schizophrenia has encouraged the examination of antipsychotic drug treatment effects on glutamate receptors. Complex region and drug type specific alterations in the expression of various AMPA and kainate receptor subunits have been reported in the rat hippocampus following haloperidol and clozapine administration (Meador-Woodruff et al., 1996). Acute and chronic antipsychotic drug treatments appear to have differential effects of NMDA receptor expression. Haloperidol (acute or chronic) appears not to alter the mRNA expression of NR1 subunit of the NMDA receptor (Riva et al., 1997; Toyoda et al., 1997) while the corresponding protein has been observed as increased (Fitzgerald et al., 1995). NR2B subunit mRNA is decreased by acute but not by chronic haloperidol treatment in whole brain extracts (Toyoda et al., 1997), whereas an increase has been detected in the hippocampus and nucleus accumbens (Riva et al., 1997). Chronic clozapine reportedly increases NR3C expression in the frontal cortex and nucleus accumbens (Riva et al., 1997). Ligand binding assays have shown that NMDA receptor binding is increased by acute haloperidol in the striatum, and after chronic treatments with haloperidol and clozapine in the cortex (Ossowska et al., 1999). In addition to regulation of glutamate receptors, glutamate transporter 1 has been reported to decrease by chronic administration of clozapine (Melone et al., 2001).

Single gene studies have indicated several other genes as regulated by antipsychotic drug treatments. The length of the drug treatment period has an impact on gene expression,
for example, mRNA of nuclear transcription factor thyroid hormone receptor alpha 1-3 is decreased by acute administration of clozapine in the shell of nucleus accumbens whereas chronic treatment increases it in the same region (Langlois et al., 2001). Expression of neurotensin, an endogeneous antipsychotic-like peptide, by acute haloperidol and clozapine follows a manner similar to that of c-Fos protein: haloperidol induces neurotensin in the striatum, and both drugs increase the mRNA in the shell of nucleus accumbens (Merchant et al., 1992; Merchant and Dorsa, 1993). Long-term haloperidol treatment increases also neurotensin protein levels in the striatum (Kinkead et al., 2000). Other genes with reported alterations in their expression by chronic antipsychotic drug treatments include several nuclear orphan receptors (Langlois et al., 2001; Werme et al., 2000), various neuropeptides (Lindefor et al., 1986; Salin et al., 1990; Zachrisson et al., 2000), and possibly several synaptic proteins (Eastwood et al., 1994; Eastwood et al., 2000; Nakahara et al., 1998).

High-throughput gene expression analyses of the clinical impact of antipsychotic drug medication on human gene expression are difficult to perform. Therefore, model systems are used for evaluation of antipsychotic drug specific gene expression. The effects of clozapine treatment have been recently examined in mouse brain using TOGA (Thomas et al., 2001a). Differentially expressed genes in striatal samples were identified after acute and long term clozapine treatments, and the expression pattern of apolipoprotein D mRNA was further examined. Expression of apolipoprotein D mRNA was increased by clozapine treatment, whereas haloperidol treatment had no effect on apolipoprotein D expression. Further analysis of apolipoprotein D levels in schizophrenia has revealed decreased protein levels in serum samples, whereas apolipoprotein D appears increased in the PFC and caudate in both schizophrenia and in bipolar disorder (Thomas et al., 2001b). However, the specific functions of apolipoprotein D in the central nervous system and in psychiatric illnesses remain unclear.

Although the immediate targets for antipsychotic drug action have been identified, it is likely that regulation of neurotransmitter receptors and transcription factors influences the expression patterns of other target genes. Only a few such candidate target genes have been identified. Large-scale analyses of antipsychotic drug effects on gene expression would help to identify pathways involved in the drug action. Such experiments would also help to interpret the gene expression patterns observed in schizophrenic patients on medication, and would potentially identify pathways involved in pathogenesis of schizophrenia. Furthermore, identification of genes targeted for regulation by antipsychotic drug medication would advance drug development efforts.
3 AIMS OF THE STUDY

Antipsychotic drugs rapidly bind to several neurotransmitter receptors and activate various brain regions in response to acute administration. Both antipsychotic and psychotropic drugs activate the limbic regions of the cortex, such as the prefrontal, anterior cingulate, and entorhinal cortices as detected by the expression of fos and jun family gene transcription factors. Although neurotransmitter receptor binding by antipsychotic drugs is acutely needed, the clinical efficacy of antipsychotic drug treatment develops only after prolonged treatment periods. Thus, there may exist other secondary targets for the drug action. The aims of the current study were to:

1. Further characterize the induction of fos and jun gene family transcription factors by acute administration of MK-801, a non-competitive NMDA receptor antagonist, in the rat entorhinal cortex.
2. Examine the effects of acute and chronic haloperidol and clozapine treatments on the expression of several fos and jun family genes in rat brain.
3. Establish the search for potential candidate target genes for antipsychotic drug action in a cell line model using DNA expression arrays.
4. Identify potential candidate target genes for acute clozapine treatment in rat brain using DNA expression arrays.
5. Examine the expression of candidate target genes for antipsychotic drug action in rat brain after chronic administration of haloperidol and clozapine using in situ hybridization.
4 EXPERIMENTAL PROCEDURES

Experiments were carried out with either PC12 cell line cultures in vitro or with rats in vivo. PC12 cell line was used in publication II, and rats were used in publications I, III and IV. All animal studies were performed in accordance with the guidelines of the Society for Neuroscience and were accepted by the Experimental Animal Ethics Committee of the University of Kuopio.

4.1 PC12 CELL CULTURE

Rat pheochromocytoma PC12 cells (publication II) (Greene and Tischler, 1976) were cultured at high density on cell culture plates coated with rat collagen. Cells were maintained in RPMI 1640 medium (Gibco BRL, Gaithersburg, USA) supplemented with 5 % fetal bovine serum, 10 % horse serum, 2 mM glutamate, and 100 U of penicillin-streptomycin. Control cultures were not treated, whereas the cultures exposed to 1 μM chlorpromazine (Sigma, St.Louis, USA) were treated for five consecutive days beginning from the day of plating. On the third day after plating, half of the culturing medium was replaced and half of the chlorpromazine dose was added for drug-treated cultures. Five days from the plating, the cultures were briefly rinsed with 2 ml of ice cold phosphate buffered saline (PBS) (pH 7.4) and the mRNA was extracted (see section 4.7).

4.2 ANIMALS

Male Wistar rats (~ 200 g, National Laboratory Animal Centre, University of Kuopio) were housed 3-6 per cage and kept under standardized temperature, humidity, and lighting conditions with free access to food and water. All drugs were administered with intraperitoneal (i.p.) injections. After drug treatments, the animals were anesthetized with CO₂ and decapitated, except for immunohistochemistry, for which the animals were administered with an overdose of pentobarbital and trancardially perfused with buffered 4 % paraformaldehyde (PFA).
4.3 DRUG TREATMENTS

In publication I, rats were given a single injection of MK-801 (5 mg / kg, Dizocilpine maleate, RBI, Natick, USA) or saline and sacrificed after 4, 8, or 24h for electromobility-shift assays, or after 4h for immunohistochemistry, and after 5h for in situ hybridization. In publication II, chlorpromazine was dissolved in H₂O, and added to PC12 cell cultures in a small volume at the final concentration of 1 μM for 5d, after which gene expression profiles of control and drug-treated cultures were established. In publication III, animals were given a single clozapine (25 mg / kg, Leporex, Wander Pharma, Novartis Pharma GmbH, Nürnberg, Germany) injection and sacrificed 1, 6, or 24h later for gene expression profiling. Control animals were injected once with saline and sacrificed after 24h. After appropriate treatment periods, RNA was extracted (see section 4.9). Chronic antipsychotic drug treatments in publications III and IV were performed by daily injections for 17d with clozapine (25 mg / kg), haloperidol (1 mg / kg, Serenase, Orion, Finland, diluted in saline), or saline. For in situ hybridization, the animals were given washout periods of 2h (17d 2hw), 24h (17d 24hw), or 6d (17d 6dw) after chronic treatment before sacrifice. In electromobility-shift assays, clozapine and haloperidol treatments were performed as described above, and saline treated controls were injected for 17d with 24h washout period.

In addition, acute treatments with haloperidol (1 mg / kg) and clozapine (25 mg / kg) were performed by giving a single injection of either drug, and the animals were sacrificed 1h later.

4.4 IN SITU HYBRIDIZATION

In situ hybridization was used to determine the mRNA expression of fos and jun family genes (publications I and IV, and unpublished data), and differentially expressed candidate genes (publication III). After appropriate treatment periods, the brains were collected, frozen on dry ice, and stored at −75°C until cut into 14 μM thick sections in coronal orientation with a cryostat. Sections were mounted on Superfrost microscope slides (Mentzel Gläser, Germany) with one control treated section in addition to one or more drug treated sections on each slide. The sections were postfixed with 4 % PFA, dehydrated, and stored at +4°C under ethanol until used.

Gene-specific anti-sense oligonucleotide probes (35-mer) were designed after sequence comparison to public databases to minimize non-specific hybridization.
Oligonucleotide probes diluted into a concentration of 0.3 pmol / µl in sterile H₂O were labeled with the incorporation of α[³²P]dATP (New England Nuclear Inc., Boston, USA) by terminal deoxynucleotidyl transferase (MBI Fermentas, Vilnus, Lithuania). Unincorporated nucleotides were removed with column chromatography using ProbeQuant™ G-50 Micro Columns (Amersham Pharmacia Biotech, Piscataway, USA). Sections were hybridized with 1-3 x 10⁶ cpm / µl of labeled probe in hybridization buffer containing 50 % formamide, 10 % dextran sulphate, and 4 x standard saline citrate (SSC) at +42°C in a humidified chamber overnight. A total of 100 µl of hybridization solution with the labeled probe was used per slide. After incubation, the sections were washed by dipping into 1 x SSC at room temperature, washed for 30 min at +55°C in 1 x SSC, washed serially for 3 min each at room temperature in 1 x SSC, 0.1 x SSC, 70 % ethanol, and finally for 5 min in 94 % ethanol. Sections were air dried and opposed onto Hyperfilm-βmax films (Amersham Pharmacia Biotech) from one day up to three weeks. Films were developed for 5 min in D-19 (Kodak, Rochester, USA).

In situ autoradiograms were quantified with video-based MCID™ image analysis software (Imaging Research Inc., St. Catharines, Canada). The quantified brain regions were determined according to rat brain atlas (Paxinos and Watson, 1986). In publications III-IV, superficial and deep cortical layers were quantified separately. Density values from the hybridized sections were converted to specific binding (nCi / g) using simultaneously exposed ¹⁴C microscales. Mean values from drug treated animals were converted to percent of mean values of control treated animals.

4.5 IMMUNOHISTOCHEMISTRY

In publication I, immunohistochemistry was performed in order to compare Fos-protein expression by MK-801 and saline treatments. Transcardially perfused rat brains were removed and post-fixed over night in 4 % PFA / PBS at +4°C, and thereafter cryoprotected in 20 % sucrose over night at +4°C. Brains were stored at −75°C until cut. Free floating 30 µm thick sections were cut with a microtome and stored in buffer containing 30 % ethylene glycol and 25 % glycerin in 0.05 M phosphate buffer (pH 7.4) at -20°C. For use, the sections were washed first 3 x 10 min in 0.1 M phosphate buffer (pH 7.4) and then in 0.1 M PBS (pH 7.4), after which they were permeabilized for 30 min at room temperature in permeabilization buffer (1 % Triton X-100 and 3 % heat-inactivated fetal bovine serum in PBS). Anti-Fos
antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, USA) recognizing rat c-Fos, FosB, Fra-1, and Fra-2 proteins was used at 1:2000 dilution in 1 % Triton X-100 / 1 % fetal bovine serum / PBS for over night at +4°C. Avidin-biotin conjugated secondary antibody (Vectastain, Vector Laboratories, Burlingame, USA) and diaminobenzidine precipitation (Zymed Laboratories Inc., San Francisco, USA) were used to visualize anti-Fos antibody immunoreactivity. Sections were then mounted onto microscope slides.

4.6 ELECTROMOBILITY SHIFT ASSAY

DNA-binding activity of the AP-1 complex was studied in publication I in the entorhinal cortex and hippocampus, and in publication IV in the prefrontal – anterior cingulate cortex preparation using electromobility shift assay. The tissues were homogenized in hypotonic buffer (10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM (p-aminophenyl)methanesulfonyl fluoride (PMSF), 1 mM dithiotreitol, 1 μg / ml leupeptin, and 1 μg / ml aprotinin). Nuclear proteins were extracted using a mixture of low-salt buffer (20 mM Hepes (pH 7.9), 25 % glycerol, 1.5 mM MgCl₂, 1.2 mM KCl, 0.2 mM ethylene-diaminetetraacetic acid (EDTA), 0.2 mM PMSF, 1 mM dithiotreitol, 1 μg / ml leupeptin, and 1 μg / ml aprotinin) and high-salt buffer (same as low-salt buffer but with 20 mM KCl) in a ratio of 2:1. Nuclear proteins were aliquoted and stored at –75°C. A consensus oligonucleotide for AP-1 (Promega, Madison, USA) was labeled with γ[32P]ATP (New England Nuclear) using T4 polynucleotide kinase (Promega). Labeled AP-1 oligonucleotide was purified using Pharmacia Microspin columns (Amersham Pharmacia Biotech). The binding reaction was performed with 5 μg of nuclear proteins and labeled AP-1 oligonucleotide in buffer containing 10 mM Tris/Hcl (pH 7.5), 5 % glycerol, 1 mM MgCl₂, 50 mM NaCl, and 0.5 mM EDTA. Poly dI/dC (2 μg, Amersham Pharmacia Biotech) was added to inhibit non-specific DNA binding. DNA-protein complexes were allowed to form for 15-20 min on ice and were resolved by 4 % polyacrylamide gel electrophoresis at 100 V for 1.5h in Tris / glycine buffer (pH 8.5). The gels were dried and exposed to X-ray films or Phosphor Screens (Molecular Dynamics Inc., Sunnyvale, USA). DNA-binding activity was quantified from X-ray films using MCID™ image analysis software (Imaging Research Inc.), and Phosphor Screens were scanned using STORM 860 Phosphoimager (Molecular Dynamics). The results are converted to percent of AP-1 binding in drug treated animals in comparison to protein extracts from saline-treated animals.
In publication IV, supershift assays were performed using antibodies raised against Fos family proteins (c-Fos, FosB, Fra-1, and Fra-2; catalog number sc-253 X, Santa Cruz Biotechnology Inc.) and Jun family proteins (c-Jun, JunB, and JunD; catalog number sc-44 X, Santa Cruz Biotechnology Inc.). Antibodies (2 μg) were added to the nuclear protein extract prior to addition of labeled AP-1 oligonucleotide, and incubated for 2h at +4°C in a rotator. Otherwise, the rest of the supershift assay was performed as described above.

4.7 NORTHERN BLOTTING

In publication II, the expression levels of differentially expressed candidate genes in PC12 cell cultures in response to chlorpromazine treatment were further confirmed using Northern blotting analysis. The cultures were briefly rinsed with ice cold PBS, and 2 ml of TRIzol™ reagent (Gibco BRL) was used to extract total RNA according to the manufacturer’s instructions. Eight μg of total RNA per lane was resolved with 1 % agarose-formaldehyde gel analysis. The gel was blotted onto Nylon membrane (Hybond N, Amersham Pharmacia Biotech) using overnight capillary blotting. The resulting membranes were hybridized with candidate clone-specific probes that were generated from plasmid DNA isolated by the alkaline lysis method (Sambrook J, 1989). cDNA inserts were excised from pBluescript SK-plasmids (Stratagene, La Jolla, USA) using EcoRI and Xhol restriction enzyme digestion (MBI Fermentas) and the digested reaction components were resolved on 1 % agarose gel. cDNA inserts were eluted from the gel using Qiaex DNA gel extraction kit (Qiagen, Chatsworth, USA). Plasmid inserts were sequenced to verify their identity and labeled for use as probes for the northern blotted membranes using Ready-To-Go random priming DNA labeling kit (Amersham Pharmacia Biotech) with the insertion of α[32P]-dCTP nucleotide (New England Nuclear). The membrane hybridization was performed in a hybridization buffer containing 0.5 M NaPO₄ (pH 7.0), 1 mM EDTA, 7 % sodium dodecylsulphate (SDS), and 0.5 % bovine serum albumin over night at +60°C. Hybridized membranes were first rinsed in 2 x SSC / 0.1 % SDS, and then washed for 40min in the same buffer at +58°C. Moist membranes were exposed onto a Phosphor Screen (Molecular Dynamics) that was scanned using STORM 860 Phosphoimager (Molecular Dynamics).
4.8 EXPRESSION ARRAYS USED IN THE STUDY

In publication II, differentially expressed genes in PC12 cell cultures treated with chlorpromazine were searched using a cDNA filter array containing 18,432 randomly selected cDNA clones from a rat entorhinal cortex cDNA library (Incyte Genomics, formerly Genome Systems, St. Louis, USA). Duplicate bacterial colonies had been spotted onto the filter, lysed to release the plasmid DNA, crosslinked, and denatured to yield single stranded DNA. Each spot represents approximately $1 \times 10^6$ copies of the plasmid.

In publication III, genes differentially expressed in the rat prefrontal – anterior cingulate cortex samples were identified using ATLAS Rat 1.2 filter array (Clontech, Palo Alto, USA) containing 1176 sequence verified PCR-generated cDNA fragments in single spots.

4.9 EXPRESSION ARRAY PROCEDURES

In publication II, mRNA was extracted from control and chlorpromazine-treated PC12 cell cultures. Cells were briefly rinsed with ice cold PBS and mRNA was extracted using QuickPrep Micro mRNA purification kit (Pharmacia Biotech). mRNAs were reverse transcribed to cDNA with SuperScript™ II RNase H reverse transcriptase enzyme (Gibco BRL) using oligo(dT) primers. Simultaneously, $\alpha^{35}$S]-dCTP (New England Nuclear) was incorporated. Following the precipitation of the cDNA pellet, RNA was digested with sodium hydroxide treatment. Prior to the addition of the resulting cDNA probes for hybridization, the filter arrays were prehybridized for 30-60 min at +55°C in hybridization buffer containing 0.5 M sodium phosphate (pH 7.0), 1 mM EDTA, 0.5 % bovine serum albumin, and 7 % SDS. A total of 500,000 cpm of the denatured control treated probe was added and hybridized for 20h at +55°C in a rotator. The hybridized filter array was washed twice in 1 x SSC / 0.1 % SDS for 15 min at room temperature and then for 1h at +55°C in the same buffer. The moist filter was wrapped in plastic and exposed to X-ray film for 4d. After exposure, the filter array was stripped by incubation in 0.2 M NaOH for 2h at +55°C, and rinsed twice in 1 x SSC / 0.1 % SDS. The stripped array was rehybridized with the probe derived from the chlorpromazine treated cells as described above. X-ray films were digitized with video-based MCID™ image analysis software (Imaging Research Inc.) for data analysis.

In publication III, brains were collected from three controls and three clozapine injected animals in each treatment group (1, 6, and 24h treatment groups) and the region
comprising the prefrontal and anterior cingulate cortices was dissected for gene expression analysis. Brain tissues were flash frozen at the time of dissection and stored at −75°C. Total RNA was extracted from pooled and homogenized tissues using Atlas™ Pure Total RNA Labeling System (Clontech) utilizing phenol-chloroform extraction according to the manufacturer’s recommendations. Total RNA was treated with DNase I, and denaturing gel analysis and absorbance measurements at A_{260}/A_{280} were used to assess the quality of RNA. Total RNA samples exhibited A_{260}/A_{280} ratios of 1.8 or higher with no degradation detected by gel analysis. Poly A+ RNA was enriched from DNase-treated total RNA samples by oligo(dT) separation followed by the cDNA probe synthesis using gene specific cDNA Synthesis Primer Mix (Atlas™ Pure Total RNA Labeling System, Clontech). Simultaneously, α[^32]PdATP (New England Nuclear) was incorporated to the synthesized cDNA probes by MMLV reverse transcriptase. Unincorporated nucleotides were removed by column chromatography according to the manufacturer’s protocol. cDNA probes from 24h saline treated control animals, and from 1, 6, and 24h clozapine treated animals were used to hybridize an Atlas Rat 1.2 Array filter. Each experiment compared the expression profiles of 1, 6, or 24h clozapine treated samples to a 24h saline treated control sample. For hybridization, the expression array was prehybridized for 30 min at +68°C in the hybridization buffer supplemented with denatured sheared salmon sperm DNA, both provided by the manufacturer (ATLAS Rat 1.2 Array, Clontech). The denatured probe was then added to the prehybridization buffer, and the filter arrays were hybridized for 19h at +68°C with continuous rotation. After hybridization, arrays were washed with continuous rotation first for four times for 30 min at +68°C in 2 x SSC / 1 % SDS, and then for 30 min in 0.1 x SSC / 0.5 % SDS, and finally for 5 min at room temperature with 2 x SSC. Moist hybridized filters were wrapped in plastic and exposed onto Phosphor Screen (Molecular Dynamics) and scanned with STORM 860 phosphoimager (Molecular Dynamics) at 50-μm resolution. The filter arrays were stripped and rehybridized according to recommended protocols in order to compare the expression profiles of all samples under study.

4.10 EXPRESSION ARRAY DATA ANALYSIS

In publications II and III, digitized filter array images were analyzed using Array Vision™ software (Imaging Research). In publication II, the signal intensity values for each cDNA spot were derived by multiplying pixel density values by the pixel area. Average background intensity values were measured locally from the surrounding of each primary grid
element (4 x 4 spots), and the intensity values of the cDNA spots were corrected by this value. Hybridization signal intensity values were calculated with the following algorithm: \((X_1 + n) / (m \times X_2 + n)\), where \(X_1\) = an average intensity value of double spots in hybridization 1 (control treated cells), \(n = 20\%\) of filter mean pixel intensity values of hybridization 1, \(m\) = ratio of filter mean pixel intensity values of hybridization 1 over hybridization 2 (chlorpromazine treated cells), and \(X_2\) = an average intensity value of double spots in hybridization 2. \(n\) was added to the \(X\) values in order to eliminate high ratios due to a low value of denominator, and \(m\) was used to normalize for the differences in hybridization conditions and probe activities.

In publication III, hybridization intensities of cDNA spots were quantified by placing a grid of 14 x 14 squares on each of the six subgrids in the filter array. Background intensity values were measured from the width of five pixels in the region surrounding each of the 14 vertical columns within a subgrid. The background-subtracted measurements of each cDNA spot intensity value were used for further analysis. Data were globally normalized by scaling the average intensity of control and clozapine treated measurement groups to the same value. The following algorithm with a correcting factor was used to compensate for increased variance among ratios calculated from small values: \(\text{ratio} = (x_{\text{sample}} + k) / (x_{\text{control}} + k)\), where \(x\) stands for the normalized measurement for a particular gene, and \(k\) is a constant value of 0.2. The size of \(k\) was defined by plotting the log-base 2(ratio) against average with different \(k\) values. Similar scatter plots have been applied previously for identification of differentially expressed genes (Dudoit et al., 2000). It was observed that 0.2*average of normalized measurements reduced the strong variation among the small measurements while not affecting the ratio values among the bigger measurements. This decreases the effect of the additive error. These corrected ratios were used to search for potentially differentially expressed genes, and were converted to log-base 2 values for data visualization with self-organizing maps. The algorithms applied in publications II and III were designed by a bioinformatician.

4.10.1 DATA CLUSTERING AND VISUALIZATION WITH SELF-ORGANIZING MAP ALGORITHM

In publication III, gene expression patterns formed by the log-base 2-transformed ratios of three time points (1, 6, and 24h) of clozapine treated samples over control treatment were further analyzed using the Self-Organizing Map (SOM) algorithm (Kohonen, 1997) (Visual Data, Visipoint Oy, Finland) which has previously been shown to be applicable for
analysis of gene expression data (Tamayo et al., 1999; Törönen et al., 1999). In the SOM analysis, the genes are clustered into a predefined number of nodes by the similarities of their expression profiles, and are visualized as a two-dimensional map. The form of the SOM is thus dependent on the data. The expression profiles of the nodes change gradually and neighboring nodes have the most similar expression profiles. Accordingly, the further away the nodes are, the more different are their expression profiles.

The distribution of genes related to predefined functional classes on the SOM map was evaluated. Expression profiles of any predetermined group of genes within the nodes can be visualized, which provides a tool to identify clusters of nodes where genes with similar expression profiles are enriched. In this study, the distribution of functional gene classes of gamma-amino butyric acid receptors (GABA) and genes related to GABA neurotransmission, glutamate receptors and genes related to glutamate-mediated neurotransmission, G-protein coupled receptors, genes involved in lipid metabolism, and genes related to presynaptic function (PSYN) were investigated. Gene groups were either based on the gene classification provided by the array manufacturer, or designed according to previous literature. These functional classes were used only for data visualization and were not involved in the clustering of the data. After visualization, clusters of nodes were defined based on the enrichment of PSYN class genes in any region in the SOM. Statistical calculation, sampling without replacement (Arnold, 1990), was used to calculate approximately the probability for finding a correlation between a cluster and a functional gene class as a result of random sampling. Statistical significance (P value) was calculated for the observed gene clusters using a cumulative hypergeometric distribution function similar to the analysis of contingency tables using Fisher exact test (Agresti, 1992). Expression array data analyses were designed by a bioinformatician.

4.11 STATISTICAL ANALYSIS

Data analysis for in situ hybridization was performed using analysis variance (ANOVA) and / or Student’s t-test (publications III and IV). For electromobility shift assay, the data were analyzed using Student’s t-test, or Dunnett’ s multiple comparison test (publications I and IV). Data of the DNA array expression analyses (publications II and III) were analyzed as described in section 4.10.
5 RESULTS

5.1 MK-801 INDUCES FOS AND JUN EXPRESSION AND AP-1 BINDING IN THE RAT ENTORHINAL CORTEX

NMDA-receptor blockade by MK-801 (5 mg / kg) greatly induced the DNA-binding activity of the AP-1 complex in the rat entorhinal cortex after 4h treatment (I, Fig 1A). After 8h, the DNA-binding activity had declined to the levels of control treatment and remained unaltered 24h following the injection. In the hippocampus, MK-801 treatment did not induce AP-1 binding at any time point (I, Fig 1B).

Next, the composition of the induced AP-1 complex was studied in the entorhinal cortex using in situ hybridization following 5h of MK-801 administration. Expression of two members of the Jun family genes were studied, c-jun and junB. mRNA level of junB was increased in the entorhinal cortex by MK-801 injection (I, Fig 2), whereas c-jun expression was unaltered (I, data not shown). In situ hybridization of the Fos family genes (c-fos, fosB, fra-1, and fra-2) revealed that c-fos, fosB, and fra-2 mRNA expression was also increased in response to MK-801 injection (I, Fig 2). Expression of fra-1 was not changed (I, data not shown).

To study whether the induction of Fos family gene mRNAs correlated with the Fos protein expression, an immunohistochemistry staining recognizing all Fos family members was performed following 4h of MK-801 treatment. Increased staining of Fos-positive nuclei was observed in the entorhinal cortex, especially in the layer III (I, Fig 3). In addition, some Fos-positive nuclei were present in the layer VI of the entorhinal cortex.

5.2 CHLORPROMAZINE INDUCES DIFFERENTIAL GENE EXPRESSION IN PC12 CELLS

A differential gene expression pattern was observed in PC12 cells treated with 1 μM chlorpromazine. Using rat entorhinal cortex cDNA library based filter array (18,432 cDNA clones), analysis of one sixth of the filter (3072 cDNA clones) revealed >300 candidate clones differentially expressed by 5d chlorpromazine treatment. One sixth of the filter was chosen for analysis because it already produced a high number of potential candidate genes. Using data analysis and visual examination, five differentially expressed clones were selected for further examination. The five selected clones were expressed at lower levels in control cells in comparison to chlorpromazine treated cells (II, Fig 2).
Sequencing of the five candidate clones revealed that two clones were cytochrome c oxidase, and three were novel genes. The expression of the novel clones, and one clone containing cytochrome c oxidase gene were further studied with northern blot analysis. Two independent northern blot studies confirmed the increased expression of cytochrome c oxidase (+26 %), and the clones unknown 1 (GenBank accession # AF250231) (+22 %), unknown 2 (GenBank accession # AF250230) (+14 %), and unknown 3 (GenBank accession # AF250232) (+42 %) (II, Fig 3) in response to chlorpromazine treatment. As of February 2002, the unknown clones have not shown significant homology to any other entries in the GenBank.

5.3 ACUTE CLOZAPINE AND HALOPERIDOL TREATMENTS AND GENE EXPRESSION IN RAT BRAIN

5.3.1 FOS AND JUN FAMILY TRANSCRIPTION FACTORS

*In situ* hybridization studies on the expression of the fos and jun family gene mRNA levels by 1h clozapine and haloperidol treatments essentially confirmed the known expression pattern of c-fos as previously described (Table 2). In addition, acute administration of clozapine induced the expression of c-fos, fra-2, and junB in the shell of the nucleus accumbens, and junB in the caudate putamen (Table 2). Decreased expression of fra-2 was observed in the infralimbic cortex, and of c-jun in the anterior cingulate cortex after acute clozapine. Acute haloperidol treatment induced c-fos expression in the PFC and anterior cingulate cortex, and increased the expression of c-fos, fra-2, and junB in the caudate putamen and nucleus accumbens (Table 2).

5.3.2 DIFFERENTIAL GENE EXPRESSION BY ARRAY ANALYSIS

Clozapine treatment for 1, 6, and 24h resulted in altered expression of a number of genes in the rat prefrontal – anterior cingulate cortex preparation of which 35 genes were selected for further analysis (*publication III*). The data from clozapine treated animals was clustered and visualized using the SOM algorithm. Data clustering using the SOM algorithm was chosen because of the experience and availability of the method in the laboratory. Data visualization revealed that the highest number of expression profiles was clustered in the node that represents no change in gene expression after any period of clozapine treatment (III, Fig
2A). Visualization of gene expression profile distribution for genes of neurotransmitter receptors (GABA and glutamate receptors), G-protein coupled receptors, or genes involved in lipid metabolism did not reveal clustering of these expression profiles (III, data not shown). Visualization of the distribution of PSYN class genes revealed enrichment in two distinct regions of nodes in the SOM. These node regions were therefore defined as clusters. In both clusters, gene expression was altered the most profoundly after 24h clozapine treatment (III, Fig 2A). In cluster A, gene expression profiles were upregulated, and in cluster B downregulated by clozapine treatment (III, Figs 2B and 2C). Statistical analysis indicated a highly significant enrichment of PSYN genes in both clusters.

Expression of three differentially regulated candidate genes, chromogranin A, synaptotagmin V and calcineurin A was confirmed using in situ hybridization. Expression of chromogranin A was modestly but consistently increased in the PFC at 6h after a single clozapine injection (III, Fig 3A). In the SOM analysis, chromogranin A was clustered in cluster A. Synaptotagmin V expression was found decreased after 24h clozapine treatment in the frontal cortex, and in the parietal cortex (III, Fig 3B). In the SOM analysis, synaptotagmin V was clustered in cluster B. In addition, array analysis suggested that the expression of calcineurin A might be induced by clozapine. In situ hybridization analysis confirmed the upregulation of calcineurin A in response to 6h clozapine treatment in the infralimbic cortex, and after 24h clozapine treatment in the frontal cortex (III, Fig 3C). These results were consistent with the filter array results.

5.4 CHRONIC CLOZAPINE AND HALOPERIDOL TREATMENTS AND GENE EXPRESSION IN RAT BRAIN

5.4.1 FOS AND JUN FAMILY TRANSCRIPTION FACTORS AND AP-1 COMPLEX

Administration of clozapine and haloperidol for 17d with varying washout periods induced the expression of several fos and jun family genes (publication IV). Clozapine treatment with 2h washout (17d 2hw) and 24h washout (17d 24hw) periods after the last drug injection had little effect on fos and jun gene expression, whereas after 6d washout (17d 6dw) period following the last drug injection the expression of several fos and jun family genes was upregulated, especially in the limbic cortex (Table 2, and IV, Figs 1 and 3). Haloperidol treatment with a 2h washout period did not induce the expression of any fos and jun family
Table 2. Expression of *fos* and *jun* family gene mRNA levels after acute and chronic treatment with clozapine or haloperidol studied with *in situ* hybridization.

<table>
<thead>
<tr>
<th>gene</th>
<th>region</th>
<th>Clozapine (25 mg/kg)</th>
<th>Haloperidol (1 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1h</td>
<td>17d2hw</td>
</tr>
<tr>
<td><em>c-fos</em></td>
<td>Fr</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>PFC</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Cg</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>IL</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>CPu</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>AcbC</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>AcbSh</td>
<td>++</td>
<td>n.s.</td>
</tr>
<tr>
<td><em>foxB</em></td>
<td>Fr</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>PFC</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Cg</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>IL</td>
<td>n.s.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CPu</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td><em>fra-2</em></td>
<td>Fr</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>PFC</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Cg</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>IL</td>
<td>n.s.</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CPu</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>AcbC</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>AcbSh</td>
<td>++</td>
<td>n.s.</td>
</tr>
<tr>
<td><em>c-jun</em></td>
<td>Fr</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>PFC</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Cg</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>IL</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>CPu</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>AcbC</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>AcbSh</td>
<td>+</td>
<td>n.s.</td>
</tr>
<tr>
<td><em>junB</em></td>
<td>Fr</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>PFC</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Cg</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>IL</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>CPu</td>
<td>+</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>AcbC</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>AcbSh</td>
<td>+</td>
<td>n.s.</td>
</tr>
<tr>
<td><em>junD</em></td>
<td>Fr</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>PFC</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>Cg</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>IL</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>CPu</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>AcbC</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>AcbSh</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Abbreviations: Fr, frontal cortex; PFC, prefrontal cortex; Cg, anterior cingulate cortex; IL, infralimbic cortex; CPu, caudate putamen; AcbC, nucleus accumbens, core; AcbSh, nucleus accumbens, shell; n.s., not significant; and n.d., not determinable. Following chronic (17d) drug administration, animals were given washout periods of 2h (17d 2hw), 24h (17d 24hw), or 6d (17d 6dw). Symbols: + or -, increase or decrease p < 0.05; ++ or --, increase or decrease p < 0.01, and ++++, p < 0.001. n = 5-6 (ANOVA followed by two-tailed Student’s t-test).
genes, while their expression was significantly increased in several brain regions 24h after the last injection (Table 2, and IV, Fig 2). Furthermore, the expression of c-fos and c-jun was increased in the limbic cortex following 6d washout period after chronic administration of haloperidol (Table 2, and IV, Fig 2).

The DNA-binding activity of the AP-1 complex was studied in the rat PFC – anterior cingulate cortex preparation after 24h and 6d washout periods following 17d administration of clozapine or haloperidol to determine the functional activity of the fos and jun family proteins. Chronic clozapine treatment with both washout periods increased the DNA-binding activity of the AP-1 complex (IV, Figure 4A and B). Chronic haloperidol treatment, in contrast, had no effect on the AP-1 binding activity at any time point (IV, data not shown). The composition of the induced AP-1 complex in response to clozapine administration was examined using a super-shift assay. Addition of Fos and Jun protein antibodies resulted in decreased AP-1 binding, and Fos antibody was able to shift the AP-1 complex (IV, Figure 4C).

5.4.2 DIFFERENTIAL EXPRESSION OF OTHER CANDIDATE GENES

Expression analysis of the acute effects of clozapine on gene expression (see section 5.3.2) resulted 35 genes which were chosen for in situ hybridization analysis in animals treated chronically with clozapine or haloperidol for 17d with varying washout periods (2h, 24h, or 6d) (publication IV). The analysis identified altered expression of several genes in various brain regions (Table 3).

Chronic clozapine administration regulated the expression of four of the analyzed genes, chromogranin A, son of sevenless (SOS), Sec-1, and VSNL-3. The expression of chromogranin A was significantly reduced in the parietal cortex after 2h washout period (~25%, p < 0.01) (Table 3 and IV, Fig 4A). At the same time point, the mRNA levels of SOS were increased in the anterior cingulate and frontal cortices (~10% and ~20%, p < 0.05 and 0.001, respectively), and decreased after 6d washout period in the PFC (~15%, p < 0.05) (Table 3). Expression of Sec-1 was increased in the parietal cortex (~20%, p < 0.05), and decreased in the PFC (~20%, p < 0.05) after 2h washout period following chronic clozapine treatment (Table 3). In addition, the expression of VSNL-3 was examined as potentially regulated. Chronic clozapine treatment with 6d washout decreased the expression of VSNL-3 mRNA in the prefrontal and infralimbic cortices (~20%, p < 0.05 and ~30%, p < 0.01 decrease, respectively, Student’s t-test) (IV, Fig 4B).
Table 3. The effects of chronic clozapine and haloperidol treatments on the candidate gene expression

<table>
<thead>
<tr>
<th>gene</th>
<th>treatment</th>
<th>Ant Cg</th>
<th>Fr cx</th>
<th>Par cx</th>
<th>PFC</th>
<th>IL cx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clozapine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChgA</td>
<td>17d 2hw</td>
<td>104.1 ± 2.4</td>
<td>91.3 ± 6.6</td>
<td>75.4 ± 6.0*</td>
<td>92.2 ± 3.4</td>
<td>103.5 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>17d 24hw</td>
<td>86.4 ± 8.0</td>
<td>91.9 ± 6.0</td>
<td>92.1 ± 4.3</td>
<td>100.9 ± 8.5</td>
<td>105.9 ± 9.0</td>
</tr>
<tr>
<td></td>
<td>17d 6dw</td>
<td>107.3 ± 7.6</td>
<td>110.3 ± 8.3</td>
<td>104.2 ± 5.5</td>
<td>101.6 ± 6.1</td>
<td>106.3 ± 3.5</td>
</tr>
<tr>
<td>SOS</td>
<td>17d 2hw</td>
<td>113.0 ± 3.8*</td>
<td>122.0 ± 2.9**</td>
<td>108.6 ± 3.3</td>
<td>110.1 ± 3.1</td>
<td>103.8 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>17d 24hw</td>
<td>82.8 ± 5.5</td>
<td>85.8 ± 6.1</td>
<td>85.9 ± 6.3</td>
<td>86.8 ± 10.3</td>
<td>83.1 ± 10.9</td>
</tr>
<tr>
<td></td>
<td>17d 6dw</td>
<td>107.1 ± 12.5</td>
<td>105.7 ± 7.7</td>
<td>105.4 ± 4.5</td>
<td>86.9 ± 3.3*</td>
<td>95.8 ± 5.9</td>
</tr>
<tr>
<td>Sec-1</td>
<td>17d 2hw</td>
<td>104.1 ± 7.2</td>
<td>98.6 ± 1.5</td>
<td>118.9 ± 4.8*</td>
<td>79.2 ± 8.2*</td>
<td>95.6 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>17d 24hw</td>
<td>101.5 ± 12.0</td>
<td>106.6 ± 11.1</td>
<td>108.5 ± 11.3</td>
<td>90.4 ± 8.2</td>
<td>105.7 ± 10.0</td>
</tr>
<tr>
<td></td>
<td>17d 6dw</td>
<td>95.7 ± 6.1</td>
<td>100.2 ± 3.8</td>
<td>98.9 ± 7.4</td>
<td>94.6 ± 3.7</td>
<td>89.9 ± 7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haloperidol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ID-2</td>
<td>17d 2hw</td>
<td>108.3 ± 9.8</td>
<td>125.0 ± 6.3*</td>
<td>99.3 ± 5.2</td>
<td>94.4 ± 8.5</td>
<td>95.2 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>17d 24hw</td>
<td>93.2 ± 2.7</td>
<td>82.7 ± 4.0</td>
<td>79.2 ± 4.3*</td>
<td>103.6 ± 8.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>Rab-12</td>
<td>17d 2hw</td>
<td>109.4 ± 4.0</td>
<td>117.7 ± 9.8</td>
<td>109.1 ± 3.7</td>
<td>123.7 ± 3.7*</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>17d 24hw</td>
<td>125.1 ± 3.7**</td>
<td>130.9 ± 3.1*</td>
<td>110.7 ± 8.2</td>
<td>99.8 ± 5.1</td>
<td>n.d.</td>
</tr>
<tr>
<td>VSNL-2</td>
<td>17d 2hw</td>
<td>56.7 ± 6.4**</td>
<td>67.1 ± 0.9*</td>
<td>58.8 ± 3.6**</td>
<td>73.0 ± 7.6**</td>
<td>75.2 ± 2.8*</td>
</tr>
<tr>
<td></td>
<td>17d 24hw</td>
<td>123.1 ± 11.6</td>
<td>124.6 ± 13.2</td>
<td>113.7 ± 11.0</td>
<td>79.3 ± 18.5</td>
<td>83.8 ± 16.0</td>
</tr>
</tbody>
</table>

All values (± SEM) are expressed as percentage of saline treated treatment period matched control sections (n = 3-6; ANOVA followed by two-tailed Student’s t-test). Abbreviations: Chga, chromogranin A; n.d., not determined. Following chronic (17d) drug administration, animals were given washout periods of 2h (17d 2hw), 24h (17d 24hw), or 6d (17d 6dw). Abbreviations: Ant Cg, anterior cingulate cortex; Fr cx, frontal cortex; Par cx, parietal cortex; PFC, prefrontal cortex; IL, infralimbic cortex. Symbols: * p < 0.05; ** p < 0.01; *** p < 0.001.

Chronic treatment with haloperidol followed by various washout periods regulated the expression of four candidate genes: inhibitor of DNA-binding 2 (ID2), Rab-12, visinin-like protein 2 (VSNL-2), and visinin-like protein 1 (VSNL-1). The mRNA expression of ID-2 was induced in the frontal cortex after 2h washout period (~25%, p < 0.05), and decreased after 24h washout period in the parietal cortex (~20%, p < 0.05) (Table 3). Rab-12 mRNA expression was increased in the PFC after 2h washout period (~25%, p < 0.05) and in the anterior cingulate and frontal cortices after 24h washout period (~25% and ~30%, p < 0.01 and 0.05, respectively) (Table 3). VSNL-2 mRNA was downregulated by chronic haloperidol treatment followed by 2h washout period in all cortical areas examined (Table 3 and IV, Fig 4C). In addition, altered expression of VSNL-3 by chronic clozapine and of VSNL-2 by
chronic haloperidol treatments encouraged us to examine the expression of VSNL-1. Chronic haloperidol treatment with 2h washout period reduced the expression of VSNL-1 in the core of nucleus accumbens (IV, data not shown).
6 DISCUSSION

6.1 THE CORTEX AS A SITE OF PSYCHOTROPIC AND ANTIPSYCHOTIC DRUG ACTION

Emotional processing appears disturbed in schizophrenia, a function that has long been suggested to engage neuronal circuits of the limbic cortex (Papez, 1937). Accordingly, non-competitive NMDA receptor antagonists that induce some of the characteristic symptoms of schizophrenia (Javitt and Zukin, 1991; Krystal et al., 1994; Lahti et al., 1995a; Malhotra et al., 1996) activate the limbic cortex both in humans (Duncan et al., 1998a; Lahti et al., 1995b; Nehls et al., 1988), and in experimental animal models (Dragunow et al., 1990; Duncan et al., 1998b; Duncan et al., 2000; Gass et al., 1993; Hughes et al., 1993; Näkki et al., 1996b; Olney et al., 1989; Olney et al., 1991; Väisänen et al., 1999). The above approach has been applied to the search for brain regions potentially involved in schizophrenia, and the knowledge has been utilized to identify possible target regions for antipsychotic drug treatment. Here, we have shown molecular correlates of activation of the rat limbic cortex by MK-801 and antipsychotic drugs. Neuronal activation in the posterior cingulate cortex by various non-competitive NMDA-receptor antagonists has been thoroughly studied, and the administration of antipsychotic drugs has been shown to at least partially abolish the activation (Farber et al., 1993; Näkki et al., 1996a; O’Neill et al., 1998; Väisänen et al., 1999). In this study, acute MK-801 treatment activated the rat entorhinal cortex, as previously described (Castrén et al., 1993; Lindén et al., 2000; Väisänen et al., 1999). Others have shown that antipsychotic drugs inhibit the neuronal activation also in the entorhinal cortex by preventing MK-801 induced expression of c-fos and BDNF in a layer-specific manner (Lindén et al., 2000; Väisänen et al., 1999). In this regard, the target brain regions for acute treatments with non-competitive NMDA-receptor antagonists and antipsychotic drugs appear at least partially overlapping.

Moreover, when administered alone, antipsychotic drugs induce neuronal activation in the limbic cortex. Expression of c-fos as a marker of neuronal activation is increased by clozapine in the PFC (Deutch and Duman, 1996; Fink-Jensen and Kristensen, 1994; Kinon and Lieberman, 1996; Robertson and Fibiger, 1992; Robertson et al., 1994; Sebens et al., 1995), whereas haloperidol appears to activate mainly striatum and nucleus accumbens (Dragunow et al., 1990; Merchant and Dorsa, 1993; Miller, 1990; Nguyen et al., 1992; Robertson and Fibiger, 1992). The effects of haloperidol in the limbic cortex have been reported more recently by other techniques than detection of IEG expression: both haloperidol and clozapine appear to trigger neurons in the PFC as detected by electrophysiological
recordings (Kim et al., 2001b), and by induced expression of transcription factors other than those belonging to fos and jun gene families (Beaudry et al., 2000). In this report, acute and chronic antipsychotic drug treatments altered the expression of fos and jun family genes, and expression of several other genes in various regions of the limbic cortex. Consistent with previous studies, acute clozapine treatment was a more potent activator of the limbic cortex than acute haloperidol treatment and it markedly increased the expression of several fos and jun family genes. Moreover, expression array analysis implicated that acute clozapine induced altered gene expression patterns in the PFC – anterior cingulate cortex. Also chronic treatments by both antipsychotic drugs induced the expression of fos and jun family genes especially in the limbic cortex. In addition, the expression of several other candidate genes was regulated after chronic administration of clozapine or haloperidol predominantly in the limbic cortex. Thus, this study together with previous reports strongly supports the role of the limbic cortex as a common site of action for non-competitive NMDA receptor antagonists and antipsychotic drugs. Nonetheless, the neuronal circuits affected by the two drug types are not necessarily identical, and the limbic cortex may represent a secondary indirect site of these actions.

6.2 USE OF EXPRESSION ARRAYS TO STUDY GENE EXPRESSION IN BRAIN

Gene expression profiling by expression arrays has revolutionized the study of single transcript analysis to simultaneous investigation of thousands of genes. Technological development of expression arrays is continuously evolving, and the rapid pace of progress is apparent also from the two expression array publications of this thesis. Whereas publication II represents the use of the first forms of the expression arrays and was an introduction to the technology, the array methodology used in publication III is more developed. Expression array technology has been applied to many fields, and the utility of the cDNA arrays in neuroscience is being evaluated.

Sensitivity of microarrays using two-color fluorescent detection has been reported to be as high as a ratio difference of 1.4-fold in a well controlled microarray study (Yue et al., 2001). Although expression arrays are semi-quantitative at their best, the majority of the studies have commonly implemented a twofold difference in expression ratio as indicative of likely altered gene expression (Watson et al., 2000). In brain, there rarely are such drastic changes in transcript expression levels (Mirmics et al., 2001b; Watson et al., 2000), which often results in low numbers of genes with differential expression, and may even falsely
identify absent gene expression (Mirnics et al., 2001b). The mRNA concentration of a low-abundance neuronal transcript that is expressed in one-third of interneurons in the cortex has been recently approximated to have an ultimate dilution factor of 18 in a microarray analysis (Mirnics et al., 2001b). Serial dilution of rare transcripts occurs through the presence of non-neuronal transcripts, transcripts from projection neurons, and by the relatively small proportion of interneurons over projection neurons in the cortex. There are also other mechanisms that may further complicate the evaluation of transcript levels in brain. Differential gene expression is likely masked if the mRNA is expressed by a large number of neurons, but it is regulated only in a subpopulation. Furthermore, neuronal populations responsive to a drug treatment may for example be sparse or widely dispersed in brain. Hence, methods to enhance signal detection in expression array studies have been developed, such as dendrimer technology, which utilizes attachment of fluorescantly labeled dendrimers into the cDNA samples under study (Stears et al., 2000). However, it is possible that the sensitivity of an expression array analysis may not be sufficient to detect small, but physiologically meaningful alterations, and reliable detection of rare transcripts in brain presents a true challenge for expression array analysis (Colantuoni et al., 2000; Marcotte et al., 2001; Mirnics et al., 2001b; Watson et al., 2000).

In this report, filter array analysis indicated altered gene expression by acute antipsychotic drug treatments in the limbic cortex of rat brain, and in the PC12 cell culture model. The results support the idea also proposed by others that the changes in neuronal transcript levels are of low magnitude. The majority of genes studied with arrays were not regulated by the treatments, or the regulation did not reach the cut-off level for differential fold-change in the data analysis. The ratio threshold indicative of differential gene expression was adjusted at the lowest to 1.4-fold in order to maximize the number of potentially regulated candidate genes. It appeared that lowering of the ratio threshold resulted in large numbers of false positive genes as evaluated by secondary screening with in situ hybridization. On the other hand, very high fold changes (three-fold and higher) by expression array analysis in individual genes were often found to be artifacts due to substantial background hybridization, and those genes were omitted from further in situ hybridization analysis. It is likely that repeated expression array hybridizations might have yielded more reliable ratio differences for individual candidate genes although the number of candidate genes would have been smaller. However, some genes were found to have expression ratios over two-fold by expression profiling that were regulated also in the secondary screening. Based on in situ hybridization analysis and northern blotting, the altered
expression often appears considerably lower in magnitude than that suggested by array analysis. For example, chromogranin A mRNA was regulated in expression analysis by over two-fold, whereas in situ hybridization study indicated an expression difference of ~15%. This could be at least partially due to concentrated signal in a single spot on the array, whereas in situ hybridization represents a more natural distribution of gene expression. It remains to be seen, whether such marginal differences in transcript expression detected by in situ hybridization have physiological consequences.

Confounding factors related to sample retrieval and RNA integrity have a central role in gene expression profiling. The accuracy in dissection of specific brain regions for expression analysis is crucial to determine genuinely altered gene expression profiles (Colantuoni et al., 2000; Watson et al., 2000). For example, inclusion of more white matter in one sample than in the other would lead to seemingly differential gene expression indicative of increased expression of glial genes and decreased expression of neuronal markers in the samples (Mirnics et al., 2001b). In this thesis work, every effort was taken to minimize such variation and the same individual in all studies performed the dissections. In addition, pooling of the brain samples from several animals within the treatment groups likely reduces variance derived from the tissue dissection. Nevertheless, expression array analysis may detect alterations due to slightly different tissue collection. One solution for this problem is the use of laser capture microscopy (Emmert-Buck et al., 1996) to collect samples from carefully chosen brain regions, or the use of RNA amplification even from single neurons (Eberwine et al., 1992). Other than tissue dissection variables, RNA composition may be affected by its quality. Use of degraded RNA in expression studies may be more informative about the stability of particular transcripts rather than of specific gene expression changes. Models using experimental animals or cultured cells in gene expression analysis, such as in this thesis, do not generally encounter such problems since RNA can be prepared fresh, whereas transcript degradation is a concern in clinical studies due to variable post-mortem periods. In this study, no indication of transcript degradation was observed in RNA samples prior to expression analysis. The composition of transcripts expressed at a given time may have been modified in in vivo studies by factors independent of drug treatment. For example, handling of animals induces a rapid and transient expression of c-fos (Sharp et al., 1991b). Here, expression profiles were determined after acute antipsychotic drug treatments and the possibility of gene expression alterations induced by handling is acknowledged. In order to minimize such variation, the control animals were handled identically with the drug-injected individuals.
Analysis of the data derived by gene expression profiling is a controversial issue regarding the methods applied. Extracting the relevant information from the available data gathered in an experiment is nevertheless crucial. Various aspects of raw data processing, that is, generating numerical data from a hybridized array image, have been explored in several publications and are not discussed here further (Mills et al., 2001; Quackenbush, 2001). From the numerical data, the simplest and the most applied form of data analysis to identify regulated genes is the calculation of expression ratios for individual genes between the samples. Although the ratio difference may indicate differential expression of several individual genes it does not allow the generation of gene expression profiles within functionally related gene groups, which are potentially more relevant for physiological responses. Characterization of networks of co-expressed genes has been suggested to be beneficial for improved understanding of various biological processes (Brown and Botstein, 1999). Methods for data clustering, such as hierarchical clustering (Eisen et al., 1998) and SOM analysis (Tamayo et al., 1999; Törönen et al., 1999) enable identification of co-regulated classes of genes and thus allow greater comprehension of large data sets (Quackenbush, 2001). Clustering methods, however, require broad previous knowledge of the genes constituting functional pathways, and such annotation is presently inadequate, especially in higher organisms. The lack of consensus in design of functional gene pathways may predispose to significantly different interpretations of the clustered data in various laboratories.

In this report, clustering and visualization of the data with SOM was applied to identify gene groups affected by acute clozapine treatment. Functional gene groups were based on the array manufacturer’s classification, or specifically designed according to previous publications. Visualization and analysis of the clustered data revealed that PSYN class gene expression profiles were clustered in discrete regions in the SOM, and that their expression was dependent on the duration of clozapine treatment. It is likely that such a group effect would not have been recognized by determination of single gene expression ratios. Clustering and visualization of the data thus allowed us to examine and identify alterations in gene expression that are potentially functionally relevant for the actions of antipsychotic drugs. On the other hand, clustering indicated several other functional gene groups that were not regulated by clozapine treatment suggesting gene group specific actions. Detection of modulated co-expression patterns of functional gene groups may open new avenues for a more detailed understanding of the biological and neurochemical basis of antipsychotic drug treatment.
Further confirmation of expression array results with independent methods is necessary, especially in identification of single genes. There are various methods applicable for secondary screening, such as real-time quantitative PCR (Bustin, 2000) and northern blotting (Alwine et al., 1977). In brain samples, these methods are vulnerable to tissue dissection artifacts mentioned above (Colantuoni et al., 2000; Mirnics et al., 2001b; Watson et al., 2000). In situ hybridization analysis can be used to localize and quantitatively analyze transcript expression also in other brain regions than those originally selected for expression analysis. Gene specific probes can be designed to any transcript if the mRNA sequence is known. In addition, sections from the same animals can be used to study the expression of several genes. In this study, in situ hybridization using a separate animal cohort and an increased number of subjects was applied to further characterize the differential expression of several candidate genes for antipsychotic drug action. Three candidate genes were found to be regulated by acute clozapine treatment. Altered gene expression was confirmed in the limbic cortex, as suggested by array analysis, and other affected brain regions were also observed. For example, expression of synaptotagmin V mRNA appeared regulated in the parietal cortex as well as in the frontal cortex. However, in situ hybridization studies did not always support the array results (Kontkanen et al., unpublished observation). Since the particular sequence of any gene applied onto the expression array was unknown in this study because of unavailability through the array manufacturer, it is possible that the oligonucleotide probes designed for in situ hybridization analyses did not detect the identical gene transcript within the expression array. On the other hand, detection of differential expression by very low expressed genes is a challenge for in situ hybridization analysis too.

Expression analysis to characterize complex neuronal diseases is perhaps one of the most intriguing applications of the methodology, and has been recently applied to resolve the molecular background of various disorders (Brown et al., 2001; Chabas et al., 2001; Hakak et al., 2001; Mirnics et al., 2000; Vawter et al., 2001; Whitney et al., 2001). However, DNA expression array analysis of complex neuronal diseases, such as schizophrenia, is particularly challenging (Colantuoni et al., 2000). A clinically identifiable phenotype may be caused by a very heterogeneous genetic composition of several abnormally functioning genes, thus complicating the interpretation of the results. Alternatively, a primary predisposing defect may not necessarily alter the overall gene expression level, or alteration may be developmentally regulated and not present in the sample. Pathophysiology can also be expressed in a different brain region than the location of the abnormal gene expression. Furthermore, potential defects in post-translational modifications as a causative factor add on
to the drawbacks of DNA array analysis. Thus, the use of proteomic techniques such as 2-dimensional gel electrophoresis together with mass spectrometric protein analysis (Pandey and Mann, 2000) and protein chip technologies (MacBeath and Schreiber, 2000) in addition to DNA arrays (Celis et al., 2000) would be warranted. Nevertheless, while many technical concerns remain in the field and need to be taken into account in the experimental design, the increasing number of reviewed publications during the last years has greatly encouraged the use of microarrays in analysis of neuronal diseases.

6.3 GENE EXPRESSION BY ACUTE MK-801 AND ANTIPSYCHOTIC DRUG TREATMENTS

Gene expression can be affected in brain rapidly, for example extracellular stimuli can induce the expression of IEG mRNA already 30 min after stimulus arrival (Herdegen and Leah, 1998). In this study, acute alterations in gene expression were studied after 1-24h treatments with MK-801 or antipsychotic drugs clozapine and haloperidol. MK-801 is known to induce c-fos mRNA already after 1h treatment, and the expression is the most profound after 4h treatment (Hughes et al., 1993; Väisänen et al., 1999). Similarly, antipsychotic drugs rapidly induce c-fos expression after 30-45 min treatment and the increase in protein level is detectable after 2h (Nguyen et al., 1992). Therefore, the selected time points for acute MK-801 and antipsychotic drug treatments applied in this report are in accordance with the time scale of approximated transcript induction periods.

Acute MK-801 administration increased the DNA binding activity of the AP-1 complex in the entorhinal cortex indicative of increased expression of fos and jun family genes. Accordingly, in situ hybridization analysis showed induced c-fos mRNA expression as previously described (Väisänen et al., 1999), and revealed increased expression of fosB, fra-2, and junB transcripts. Furthermore, c-Fos protein was upregulated by MK-801 especially in the layer III, as reported by others (Väisänen et al., 1999). Thus, the results presented here corroborate earlier studies describing activated gene expression by acute non-competitive NMDA-receptor antagonists in the limbic cortex, and further clarify the actions of MK-801 suggesting that the functional effects of MK-801 administration are on target gene expression. The actual target genes for MK-801 induced AP-1 complex, however, remain to be elucidated.

We performed a comprehensive in situ hybridization analysis of acute antipsychotic drug treatment effects on expression patterns of several fos and jun family genes, study of which has not been previously examined in such detail. Induction of c-fos by acute clozapine
and haloperidol treatments showed well defined regional patterns that are consistent with the literature (Fink-Jensen and Kristensen, 1994; Merchant and Dorsa, 1993; Nguyen et al., 1992; Robertson and Fibiger, 1992; Sebens et al., 1995). In particular, haloperidol induced the gene expression most strikingly in the striatum and in the core of the nucleus accumbens, whereas both drugs increased gene expression in the shell compartment of the nucleus accumbens. However, we were unable to detect an increase in c-fos expression by acute clozapine treatment in the PFC. Since c-fos induction has been shown in this region by a number of reports, it is possible that differences in animal strains or treatment regimens account for the difference. Nevertheless, the results implicate a widespread regulation of several fos and jun family genes in a simultaneous and coordinate manner in several brain regions by acute antipsychotic drug treatments. As Fos and Jun transcription factors dimerize for effective transcriptional regulation (Herdegen and Leah, 1998), it is likely that regulation of presently unknown target genes of antipsychotic drugs occurs in these brain regions.

In this study, we used DNA expression arrays to search potential target genes affected by acute clozapine treatment in the limbic cortex. The gene class focused approach was used to identify alterations in functional pathways in addition to single genes, since drug treatments potentially modify expression patterns of interacting networks of genes (Debouck and Goodfellow, 1999). Furthermore, the variety of genes and functional gene classes reported as affected in schizophrenia suggests that antipsychotic drug treatment likely does not alleviate schizophrenic symptoms by affecting expression of any individual gene (Hakak et al., 2001; Mirnics et al., 2000; Vawter et al., 2001). It seems more plausible that changes in the expression of genes involved in certain pathways might be related to the action of antipsychotic drug treatment, and the results presented here support this notion. Data clustering supplemented with the known gene functions revealed that acute clozapine treatment altered expression profiles of genes related to presynaptic function in the limbic cortex. Clozapine treatment had a dual effect on PSYN class gene expression: a subset of PSYN genes was induced, and another subset was decreased by acute clozapine treatments. No clear functional differences were observed on these two groups of PSYN class genes, but it makes sense that changes in an interactive biological system would not be induced only in one direction. Altered expression of genes related to synaptic signaling and connectivity has been recently proposed in schizophrenia using a microarray approach (Mirnics et al., 2000; Vawter et al., 2001), and single gene studies support this hypothesis (Eastwood and Harrison, 2001). Furthermore, there is evidence for the capability of antipsychotic drug treatment to
balance some of the putative defects in synaptic connections in schizophrenia (Konradi and Heckers, 2001).

We further investigated the expression patterns of two PSYN class genes after acute clozapine administration using in situ hybridization, and observed altered expression profiles as suggested by the DNA array analysis. Expression of chromogranin A was increased by acute clozapine treatment in the PFC. Chromogranin A is a calcium binding protein enriched in large dense core vesicles controlling vesicle formation and secretion, and it is also secreted as a peptide (Kim et al., 2001a; Yoo, 2000). Chronic administration of clozapine has been reported to modulate chromogranin A mRNA expression in brain (Bauer et al., 2000; Kroesen et al., 1995), and another chromogranin family gene member, chromogranin C, has recently been reported to be increased in the PFC in schizophrenia (Hakak et al., 2001). Synaptotagmin V mRNA was found decreased by array analysis, and this was confirmed using in situ hybridization. Synaptotagmin V is likely involved in exocytosis of synaptic vesicles (Südhof and Rizo, 1996), and its expression has been recently found to be decreased in the PFC in schizophrenia (Mirnics et al., 2000). Finally, we confirmed the clozapine-induced expression of calcineurin A, a Ca²⁺ and calmodulin dependent serine-threonine protein phosphatase (Rusnak and Mertz, 2000). Clozapine has been suggested to inhibit calcineurin A activity (Gong et al., 1996). Furthermore, expression of calcineurin A has been recently suggested to be increased in the PFC of neuroleptic-mediated schizophrenic patients (Hakak et al., 2001). Overall, the results of acute clozapine effects on gene expression support the role of antipsychotic drug treatment in synaptic modulation. Striking similarities in the expression profiles of the above mentioned genes by acute clozapine treatment in rats and in medicated schizophrenic patients may be potentially explained by two alternative hypotheses. First, acute administration of clozapine may modulate the expression of synapse-related genes that are affected in schizophrenia, or secondly, the gene expression patterns observed in schizophrenia may be affected by antipsychotic drug treatments. The latter possibility appears perhaps more likely, since the vast majority of gene expression studies performed in schizophrenic patients, whether single gene or high-throughput studies, have been influenced by long-term antipsychotic drug treatments. Using a primate model, it has been suggested that chronic haloperidol treatment does not alter the expression of synapse-related genes (Mirnics et al., 2000). However, further studies are warranted to clarify the role of antipsychotic drug treatments in gene expression.
6.4 GENE EXPRESSION BY CHRONIC ANTIPSYCHOTIC DRUG TREATMENTS

In contrast to acute dopamine receptor antagonism that occurs immediately upon antipsychotic drug administration, long-term treatment is required for clinical efficacy to develop. Thus, effects other than receptor binding must occur during the therapeutic latency. Modification of gene expression patterns might help to explain the delayed effect. In this report, we have showed a widespread regulation of transcription factors and several other genes in response to chronic administration of clozapine and haloperidol.

Evaluation of the *fos* and *jun* gene expression patterns and the expression of other candidate genes after chronic antipsychotic drug treatments using *in situ* hybridization revealed similarities in the affected brain regions. After chronic treatments, the expression of *fos* and *jun* genes was profoundly induced in the cortex with fewer effects in the striatum and nucleus accumbens. *fos* and *jun* gene induction following chronic haloperidol treatment in the cortex, however, did not increase AP-1 binding activity, which may be explained by binding of these proteins to other sites than AP-1 sequences in DNA. Expression of the majority of other candidate genes for antipsychotic drug action was also modulated mainly in the cortex by both drug treatments. Cortical action of long-term antipsychotic drug treatments has been recently suggested (Lidow et al., 1998). In schizophrenia, molecular and morphological abnormalities in the cortex (Harrison, 1999a) correlate well with the disturbed limbic cortical functions (Carter et al., 1997; Goldsmith et al., 1997; Haznedar et al., 1997; Nordahl et al., 2001; Sabri et al., 1997; Silbersweig et al., 1995). Furthermore, chronic antipsychotic drug treatments profoundly modulate the cortical dopaminergic signaling; chronic administration of clozapine and haloperidol increases cortical D₂ receptor expression (Lidow et al., 1998; Rupniak et al., 1985; See et al., 1990), and induces a depolarization block in the majority, but not in all, of the dopaminergic projection neurons in the midbrain (White, 1996). The depolarization block is caused by sustained dopaminergic activity elicited by repeated administration of antipsychotic drugs, which produces a state of continuous neuronal depolarization. Firing activity can be returned to normal after repolarization of the membrane potential. Clozapine and haloperidol induce depolarization block in the mesolimbic and mesocortical dopaminergic projection neurons, which may have implications in cortical gene expression patterns induced by chronic treatments. Thus, our results support the substantial cortical action of long-term antipsychotic drug treatments but further experiments, such as microarray analysis of long-term antipsychotic drug treatment effects on various brain.
regions, would be beneficial for a more coherent understanding of the target regions of the treatments.

Schizophrenia has been suggested to involve a synaptic pathology, and antipsychotic drug treatments may have potential to reverse some of the synapse-associated defects of the syndrome. Therefore, it is interesting to note that several of the candidate genes regulated by chronic treatment with clozapine or haloperidol, such as chromogranin A, rab-12, and Sec-1, are involved in vesicle transport, and by virtue, potentially involved also in synaptic transmission (Carr et al., 1999; Kim et al., 2001a; Yoo, 2000; Zerial and McBride, 2001). The role of these genes in long-term antipsychotic drug action is further supported by regulated expression of chromogranin A also by acute clozapine treatment in expression array analysis, and by the altered expression of rab-12 by acute haloperidol treatment in in situ hybridization analysis (Kontkanen et al., unpublished observation). Ultrastructural synaptic modulation has been observed in rodents after chronic antipsychotic drug treatments by electronmicroscopic examination (Harrison, 1999b; Konradi and Heckers, 2001). The majority of alterations has been described in the striatum but also in the cortex, especially in the PFC. It has been reported that long-term antipsychotic drug treatment induces morphological adaptations in cellular localization and appearance of synapses, modifies the presence of structural synaptic specializations, and alters the size and number of mitochondria in axon terminals and dendrites. Cessation of chronic drug administration appears to reverse most but not all of the above-mentioned alterations while it is unclear whether the modifications are related to the alleviating effects or to the side effects produced by the treatments. These lines of evidence indicate a role for synaptic modulation by long-term antipsychotic drug treatments. The results presented in this thesis lend support to this hypothesis.

Ca\(^{2+}\)-mediated intracellular signaling is crucial for several basic functions of neurons. In this thesis, differential expression of several genes related to regulation of intracellular Ca\(^{2+}\) homeostasis by chronic antipsychotic drug treatments was observed. Especially, visinin-like proteins that belong to the family of intracellular EF-hand calcium sensor proteins (Braunewell and Gundelfinger, 1999), were regulated by chronic haloperidol and clozapine treatments. Visinin-like proteins (VSNL-1, VSNL-2 and VSNL-3) regulate many effector proteins including certain G-protein coupled receptor kinases at the cell membrane in response to changes in Ca\(^{2+}\) concentration (Braunewell and Gundelfinger, 1999; Iacovelli et al., 1999). Potential relationship between visinin-like proteins and schizophrenia has been recently implicated. VSNL-1 protein has been suggested as abnormally localized in the
hippocampus of schizophrenic patients (Bernstein et al., 2002). In addition, expression of VSNL-1 appears regulated in a phencyclidine model of schizophrenia (Kajimoto et al., 1995).

We aimed to further characterize the time scale of chronic treatment-induced alterations in gene expression, and observed that the expression of fos and jun family genes was dependent on the washout period after the last dose of chronic drug treatments, whereas the expression of other candidate target genes did not follow the washout period. Fos and jun family genes were not induced immediately after cessation of chronic antipsychotic drug treatments, but the expression increased over time from the last drug injection. The majority of the other candidate genes for antipsychotic drug action were regulated predominantly immediately after the last dose of either drug, whereas a few genes were regulated after longer washout periods following the chronic treatments. Thus, it appears that temporal regulation of fos and jun family transcription factors following chronic antipsychotic drug treatments differ from other candidate genes examined in this study. Given the different natures of these genes, simultaneous alterations in their expression would not be expected, since it is not clear which transcription factors regulate the expression of the candidate genes.

It is possible that repeated administration of clozapine and haloperidol may lead to drug accumulation in brain, and thus to result in altered gene expression several days after cessation of the treatments. The terminal half-life of clozapine has been reported to be 1.5 – 1.6h in rat brain, and it does not significantly accumulate after a week of daily i.p. dosing (Baldessarini et al., 1993). Moreover, others have shown an absence of clozapine in rat brain following chronic administration (Kuoppamaki et al., 1993; Kuoppamaki et al., 1994). Thus, it is unlikely that significant amounts of clozapine are present in brain after withdrawal periods of 24h or more. The terminal half-life of haloperidol has been estimated to be 1.5h in rat (Cheng and Paalzow, 1992), but longer half-lives have also been reported (Cohen et al., 1992). Therefore, residual amounts of haloperidol may still be present in brain several days after withdrawal of drug. The majority of the other candidate genes studied by in situ hybridization analysis were regulated early after the last of the chronic drug injections, and the altered expression was attenuated as the withdrawal period was extended. Thus, they appear potentially responsive to high antipsychotic drug levels in brain. In contrast, the expression of fos and jun family genes was the most pronounced after long withdrawal periods following chronic antipsychotic drug treatments. Given the short half-lives, it is unlikely that the presence of antipsychotic drugs was responsible for these changes. However, withdrawal from the long-term antipsychotic drug treatment in schizophrenia may result in a rapid clinical relapse in some patients (Ekblom et al., 1984; Gilbert et al., 1995; Meltzer et al.,
1996; Shiovitz et al., 1996). The occurrence of relapse in patients withdrawn from clozapine appears higher than that of patients treated with typical antipsychotics (Meltzer et al., 1996). Recent data suggest that the dissociation of clozapine from D2 receptors is faster than that of haloperidol due to weaker receptor binding, which may explain the rapid relapse (Kapur and Seeman, 2000; Kapur and Remington, 2001; Seeman and Tallerico, 1999). Clozapine withdrawal often induces a cholinergic rebound effect (Shiovitz et al., 1996) which may involve alterations in gene expression. Thus, increased expression of fos and jun family genes after substantial withdrawal periods may be associated with a drug withdrawal effect at the level of gene expression.

In this study, we also sought to determine potential target genes for long-term antipsychotic drug treatment using a rat dopaminergic PC12 cell culture model. PC12 cells have been widely applied to in vitro studies of antipsychotic drug action, and although not derived from the central nervous system, they exhibit several neuronal characteristics such as receptors for dopamine and noradrenaline, and vesicular neurotransmitter release in response to stimulation (Courtney et al., 1991; Schomig et al., 1988). Expression array analysis after chronic administration of the typical antipsychotic agent chlorpromazine demonstrated an induced expression of several potential candidate clones. The expression of five clones was further examined using northern blotting, which confirmed the results of expression array analysis. Two of the clones were cytochrome c oxidase, an enzyme whose activity has been correlated with neuronal activity (Wong-Riley, 1989). An increased transcription of cytochrome c oxidase by chlorpromazine suggests that metabolism is activated in the cultured cells. Three novel genes were also found to be regulated by chlorpromazine. The function of these genes is unknown but could be related to the action of chlorpromazine. It would be interesting to study the expression profiles of these three unknown candidate genes by antipsychotic drug treatments in rat brain. Although the complex neuronal connections of an innate brain are not mimicked by cell cultures, the data provided by cell culture models can serve as a sound basis for more complex investigations in whole animals.

6.5 APPLICATIONS TO ANTIPSYCHOTIC DRUG RESEARCH AND SCHIZOPHRENIA

Antipsychotic drug treatment produces a wide range of actions in brain. The effects are not restricted only to regions of neurotransmitter receptor antagonism, but extend also to regions receiving projections from the affected primary sites. Thus, complex neuronal circuitry is inevitably influenced. Presently, the brain regions affected by acute treatments
have been characterized in detail, whereas the regional activation by chronic treatments has
remained less well studied. The results of this report together with previous literature suggest
a pivotal role of the cerebral cortex in neuronal activation induced by chronic administration
of clozapine and haloperidol. In addition, this report has supplemented the previous literature
on the cortical effects of antipsychotic drug treatments by defining genes and brain regions
affected.

The ultimate molecular pathways affected by antipsychotic drug treatments are elusive, while the immediate pathways have now been established. Although neurotransmitter
receptor antagonism is considered to be intimately related to the clinical efficacy of
antipsychotic drug treatments, it may not be the final target for the drug action, but is likely
encompassed in networks of secondary/tertiary drug effects. This thesis suggests a potential
regulation of functionally relevant synapse-related genes by acute and chronic antipsychotic
drug treatments that is supported by previous reports. It remains to be established, whether the
altered gene expression patterns observed in this study are related to the clinically beneficial
effects or with the side effects of the treatment. Moreover, it is unclear in which
neurotransmitter systems the presynaptic genes are affected. Such knowledge would be
informative for characterization of neuronal networks affected by the drug treatments, and
might provide insight into the potential abnormalities in neuronal connectivity in
schizophrenia.

Gene expression studies in schizophrenia are inevitably influenced by long-term
antipsychotic drug administration, and thus the gene expression patterns in schizophrenics
may be largely altered by the presence of the drugs. Therefore, understanding the molecular
actions of antipsychotic drug treatments would help to distinguish the gene expression
alterations that are characteristic for the disease from those induced by drug treatments.

Finally, antipsychotic drugs available at present produce a wide variety of side
effects during long-term treatments. Characterization of gene expression patterns induced by
antipsychotic agents may help to identify new potential drug targets, and may help in the
design of better tolerated and more effective treatments.
7 SUMMARY

This study was undertaken to examine the alterations in gene expression induced by antipsychotic drug treatments in a cell culture model and in rat brain. Furthermore, gene expression after MK-801 administration was studied in the rat entorhinal cortex. The following results were obtained:

1. MK-801 treatment induced the expression of *fos* and *jun* family gene members in the entorhinal cortex, especially in the layer III. Fos protein also appeared increased in the same region. The DNA-binding activity of the AP-1 complex was induced after MK-801 administration in the entorhinal cortex, whereas no change was observed in the hippocampus.

2. Acute treatment (1h) with haloperidol and clozapine induced the expression of several members of *fos* and *jun* gene families in rat brain as expected. Chronic administration (17d) of haloperidol and clozapine with varying washout periods (2h, 24h, and 6d) induced a long-lasting expression of *fos* and *jun* family genes in the prefrontal cortex. Chronic clozapine also induced the DNA-binding activity of the AP-1 complex in the prefrontal cortex, whereas haloperidol had no effect.

3. A PC12 cell culture was applied to search for candidate target genes regulated by 5d chlorpromazine treatment using DNA expression array. A large number of candidate genes were obtained, of which the regulation of five clones was further confirmed. Chlorpromazine treatment induced the expression of cytochrome *c* oxidase and three novel genes.

4. Potential candidate target genes for antipsychotic drug action were searched *in vivo* in the rat prefrontal cortex after acute administration (1h, 6h, and 24h) of clozapine. Data clustering revealed an altered expression of genes involved in presynaptic function that was further confirmed. Expression of presynaptic genes was the most altered after 24h clozapine administration.

5. Expression of potential target genes for antipsychotic drug action was further examined after chronic (17d) haloperidol and clozapine treatments in rat brain. Chronic treatments altered the cortical expression of several genes involved in presynaptic function. Genes involved in modulation of calcium homeostasis were also found to be regulated.
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