

# Expression Profiling Methods Used In Drug Abuse Research

PETER J. GEBICKE-HAERTER

*Department of Psychopharmacology, Central Institute for Mental Health, University of Heidelberg, Mannheim, Germany*

## Abstract

*A variety of analytical methodologies to investigate gene expression patterns in cells or tissues have been developed. For screening purposes, a large number of target mRNAs have to be interrogated simultaneously. These requirements have been met more or less comprehensively by Differential Display (DD) RT-PCR, Suppression Subtractive Hybridization (SSH), Serial Analysis of Gene Expression (SAGE), and DNA chips. The ultimate goal to cover any gene transcript potentially expressed by a given cell is on the way to be achieved by microbead arrays and by Affymetrix gene chips. Once targets of interest are identified, techniques employing low degrees of multiplexing, such as RNase protection assays or some bead-based techniques (Luminex) eventually provide extremely fast results on the diagnostic level. With the aid of powerful computer programs, expression profiling technologies have opened intriguing new insights into the complex world of gene regulation. These new techniques have also been applied in drug abuse research recently and some examples of such approaches are described.*

## Introduction

Expression profiling can be a powerful means to obtain detailed insights into transcriptional changes in response to environmental and pharmacological stimuli in any given tissue or cell type. The extent of insight greatly varies with the methods to be used. Some methods are suitable for monitoring low numbers of transcripts, whereas others allow for screening of thousands of gene transcripts, simultaneously. Before a decision is made which method is to be used, it is also important to take into consideration whether only known transcripts or also as yet unknown transcripts are to be analyzed. Finally, it has to be taken into account that respective methodologies are more or less expensive. This article intends to provide a brief overview of the technical tools developed and used until now in expression profiling and describes some examples where these techniques have successfully been employed in drug abuse research.

## RNase Protection Assay

Approaches to analyze mRNA expression in biological samples date back to the early eighties when people started to quantify RNA by RNase protection (Melton et al., 1984). This technique has been further developed into a multiplex procedure and is commercially available as RPA-kits (Pharmingen-BD Biosciences, San Diego, Ca., USA). It is a solution-based hybridization technique where specific, radiolabeled (or biotinylated) cDNA probes are added to total

RNA and hybridization is driven to completion. In the subsequent reaction, any single-stranded RNA is digested leaving behind labeled RNA/DNA-hybrids. These are resolved in a polyacrylamide gel. Probes are designed in such a way that resultant hybrids have different sizes, so they can be resolved in a gel, and thus, multiplexing is feasible. There are presently dozens of kits available for a lot of research issues. The number of gene transcripts that can be subjected to simultaneous analyses, however, is limited to approximately 10–15. Nevertheless, if targets are identified, this technique provides an excellent means to accurately quantify mRNAs in a linear range covering several orders of magnitude. Since it does not rely on prior reverse transcription of source RNA into cDNA or on any amplification steps, it reflects the quantitative occurrence of mRNAs under investigation extremely well. It is used, therefore, to confirm data from DNA chip experiments (see below). RNase protection assays have also been used in addiction research. For example, NMDA receptor subunit expression has been investigated in brains of alcohol treated rats (Follesa and Ticku, 1995). Nine hours after the last ethanol administration NR2A and NR2B subunit transcripts were elevated in cerebral cortex and hippocampus, whereas the NR1 subunit was unchanged during alcohol treatment or withdrawal in each brain region examined. RNase protection assay was also applied to study the effects of acute binge cocaine on opioid peptide and CRH receptor expression in  $\mu$ -opioid receptor knock-out mice (Zhou et al., 2002). Zhou et al. detected a specific role of  $\mu$ -

---

Correspondence to: Peter J. Gebicke-Haerter, Dept. of Psychopharmacology, Central Institute for Mental Health, J5, 68159 Mannheim, Germany. Tel: +49(621)1703-6256; E-mail: gebicke@zi-mannheim.de.

opioid receptors in the caudate putamen and N. accumbens on preproenkephalin gene expression upon “binge” treatment with cocaine, but failed to observe substantial influence of these receptors on preprodynorphin expression or on HPA axis activation. Follesa and Ticku have used the technique to show differential effects of chronic ethanol treatment on NMDA receptor subunits.

### Differential Display RT-PCR

Differential display (DD) RT-PCR, first introduced by Liang and Pardee (1992), has been widely used in research including brain research in general and in drug abuse research. The procedure entails two major steps: (1) reverse transcription of mRNAs using a set of degenerate oligo dT primers and (2) PCR amplification of resultant cDNAs using random primers in combination with the original oligo dT primers. The use of radiolabeled dNTPs in combination with PCR makes it very sensitive to the effect that either low amounts of starting material are required or low-abundant transcripts are detectable. With the most recent improvements of the method, it has become possible to start with as little as 5 ng of total RNA, corresponding to approximately  $10^4$  cells. Moreover, the method is highly reproducible, relatively inexpensive, and does not rely on hybridizations. With its help, it is possible to detect entirely new genes being differentially regulated. A putative drawback is the rather low number of genes revealed by the method. Moreover, it is often very hard to obtain pure differentially expressed cDNAs from the separating polyacrylamide gels, since the radiolabeled bands of interest have to be excised. Additional cDNAs with the same or very similar sizes as the band of interest may be cut out along with the correct band and may compound subsequent sequencing results. There may also be gel shrinkage or distortion before radiolabeled bands are identified through overlay of respective x-ray films.

Yang and Zoeller (2002) have used DDRT-PCR to investigate brains from fetal rats (gestational days 13 and 16), the mothers of which had been treated with ethanol. In this study, interferon-inducible protein 10 (IP-10) was the major upregulated transcript. One consequence of enhanced IP-10 production – as shown in brain slices – is inhibition of LTP (Vlkolinsky et al., 2004). Cellular sources of IP-10 are non-neuronal cells, microglia, in particular (Ren et al., 1998), and in its function as a chemokine it is supposed to recruit leukocytes from the circulation. Ethanol, hence, can also evoke an immune reaction in the brain. In two other studies examining either brain tissue from human alcoholics or from alcohol preferring rats (Fan et al., 1999; Sommer et al., 2001), transcripts encoding ribosomal proteins like S12, S16, L18 showed up in the gels. Since often these genes are used as internal standards in Northern Blot or Real-Time PCR quantifications, it is interesting to notice that – at least under the influence of ethanol – they are not as unchanged as assumed. There is, however, another transcript in the report by Sommer et al. that needs increased attention: diacylglycerol (DAG) kinase  $\iota$ . It is a key enzyme controlling levels of DAG, an intermediate lipid inserted in the phosphatidylinositol second messenger pathway. It can stimulate protein kinase C (PKC) in this way influencing cellular growth. Since DAG kinase  $\iota$  contains two zinc finger-like motifs it can also

bind DNA and, hence, affect gene transcription. Apart from its expression in testis, DAG kinase  $\iota$  is only expressed in brain. It has been cloned very recently from rat brain (Ito et al., 2004). The authors find strong *in situ* hybridization signals in cerebellar cortex, in olfactory bulb and in hippocampus. Weaker expression is observed in cerebral cortex and caudate putamen. Two isoforms are described that are characterized by insertions that result in frameshifts and premature stop codons. Compared to the wild type form of 1050 amino acids, the two isoforms are composed of only 591 amino acids. Nevertheless, both isoforms are translated into protein, the DAG kinase  $\iota 3$  even at markedly high frequency (42 % vs. 53 % of wild type form). Moreover, the authors convincingly show that the truncated variant 3 has no enzymatic activity, whatsoever. Since the splice variants are triton-insoluble, their shuttling between cytoplasm and nucleus may be distinct from the wild type form. It may be concluded from this, that the total activity of DAG kinase  $\iota$  is dependent on the expression ratio of the splice variants and that environmental stimuli may have an influence on this expression ratio.

In the study by Peng and Simantov (2003), DDRT-PCR has been used to identify transcripts differentially regulated upon MDMA (ecstasy) treatment in mice. They found a number of genes but only took a closer look to the GABA-transporter (GAT) that was upregulated in frontal cortex and midbrain. A sustained, elevated mRNA level of GAT1, but not GAT4 was observed up to 7 days. Although MDMA affects serotonergic, dopaminergic, and glutamatergic neuronal activities, the authors point out, that attenuated GABA transmission by upregulated GAT and subsequent, increased dopamine release may be key features of MDMA action. Unfortunately, they do not dwell on the other gene transcripts in their list. Down-regulation of dystrophin transcription likely influences the GABA-ergic system, as well. Although it is often associated with  $K^+$ -channels, it is apparently involved in the regulation of GABA<sub>A</sub> receptors (Wallis et al., 2004). In this line, it has been reported earlier to subserve a function in maintaining the correct sizes of GABA<sub>A</sub> channel clusters (Anderson et al., 2003). Finally, a decrease of dystrophin expression in neurons results in lowered expression of neuronal nitric oxide synthase (nNOS), and likely in reduced production of the important retrograde messenger nitric oxide (Sogos et al., 2003). The down-regulation of the GTPase Nedd5 by MDMA may also impact on the proper function of neurons, since it is closely associated with microtubules and ensures correct transport of molecules or organelles along neuronal fibers. Its down-regulation has been shown to result in neurite degeneration (Vega and Hsu, 2003; Surka et al., 2002).

Brenz Verca et al. (2001) investigated cocaine-induced transcriptional changes by DDRT-PCR and found a new transcript, CD81, specifically induced in N. accumbens. CD81-deficient mice show altered sensitivity to cocaine as assessed with locomotor activity and place preference (Michna et al., 2001). The knock-out mice also showed higher dopamine levels in N. accumbens than controls. These and other studies (Bahi et al., 2004) suggest that CD81 expression in the mesolimbic dopaminergic pathway contributes to behavioral changes associated with cocaine sensitization. CD81 is known to be expressed in astrocytes and involved in cell adhesion.

### Suppression Subtractive Hybridization

Suppression subtractive hybridization is a technique to identify differentially expressed genes in two samples without sequence information (Diatchenko et al., 1996). It takes advantage of different rates of cDNA hybridization (competition) due to their (differential) levels of abundance. Two rounds of hybridizations are performed. In the first round, the levels of abundant and rare transcripts are normalized. In the second round, the normalized transcripts from control and experimental samples are subjected to subtraction hybridization. The transcripts remaining in the experimental fraction after subtraction are PCR-amplified, cloned into a cDNA library and sequenced. Eventually, sequencing of many inserts has to be carried out to learn more about the nature of these genes.

Jacobs et al. (2002; 2005) used suppression subtractive hybridization to investigate transcriptional changes in *N. accumbens* shell upon heroin self-administration in comparison to a yoked-control animal. The aim of the study was to find out, whether or not it makes a difference to voluntarily (self-) inject a drug or receive a forced (non-contingent) injection. To this end, two rats were wired with jugular vein catheters with one animal allowed to self-inject heroin by nose poking, whereas the other animal received unavoidably the same amount of drug at same frequencies as done by the self-administering rat. Transcriptional changes were strikingly different in these two animals. In self-administering rats, the majority of genes were down-regulated, whereas in the control rats there were only few changes. Amongst these, cAMP-regulated phosphoprotein 21 (ARPP-21), protein 14-3-3, and glial cell line-derived neurotrophic factor (GDNF) were significantly down-regulated in self-administering animals but significantly upregulated in yoked-controls. In their subsequent publication, the authors report, that screening of the same genes in *N. accumbens* core revealed no qualitative differences (Jacobs et al., 2005) and conclude, that cognitive processes associated with drug self-administration direct long-term responses in the shell, whereas pharmacological effects of addictive drugs are primarily mediated by the core.

### Serial Analysis of Gene Expression (SAGE)

Serial Analysis of Gene Expression (SAGE) is another method to study gene expression in different samples without prior knowledge of target genes (Velculescu et al., 1995, 1997). In contrast to RNase protection assays or suppression subtractive hybridizations, it is not dependent on hybridizations. All oligo dT-primed, reverse-transcribed cDNAs are cut down to lengths of 14–20 bp and linked tail-to-tail into “di-tags”. Concatemers of 25–75 di-tags are cloned into a library and sequenced. In this way, individual clones contain inserts representing more than one gene. Subsequent sequencing, hence, yields information about multiple genes per clone insert and markedly reduces the number of sequencing reactions. On an average, this reduction is 30–50-fold, which means that instead of sequencing 50,000 clones, only 1,700–1,000 have to be done. The fundamental drawback observed here and in other techniques has been the dependence on cDNA fragments located in the 3'-untranslated tail of the mRNAs. This problem has successfully been tackled recently by modifying the original procedure in that no oligo-dT primers were used for reverse transcription but random 12–

33-mers (Dias-Neto et al., 2000; Camargo et al., 2001). The method has been named “open reading frame expressed sequence tags” (ORESTES) and turned out to display stretches of cDNA covering all parts of the translated part of mRNAs. The data obtained by SAGE compared to data from e.g. Affymetrix chips (see below) revealed some differences in up- or downregulated genes. High- and medium abundant transcripts are reliably detected with both techniques, whereas results from low-abundant mRNAs are inconsistent (Evans et al., 2002). Possibly, SAGE provides more reliable quantitative results with low-abundant transcripts (Menssen & Hermeking, 2002). Until now, SAGE technology has not been applied to drug abuse research.

### Total Gene Expression Analysis (TOGA)

TOGA, which has first been developed by Sutcliffe et al. (2000), involves oligo-dT-primed reverse transcription of mRNAs into cDNAs, restriction endonuclease digestion and cloning 3'-cDNA fragments into an expression vector. This library is *in vitro* transcribed into cRNA and again reverse transcribed into cDNAs. These are PCR-amplified and labeled by incorporation of one fluorescently labeled primer. The PCR products are resolved on a polyacrylamide gel and fluorescence signals are quantified (for further details, see Spence et al., 2005). This method has been combined with quantitative trait loci (QTL) analyses. Chromosomal locations already identified to segregate with e.g. alcohol preference are matched with candidate genes showing up in TOGA. This reduces the number of candidate genes but provides additional strong indication that the “match-winners” are excellent candidates.

Spence et al. (2005) have screened rats selectively bred for alcohol-preferring (P) or non-preferring (NP) using TOGA technology. At the same time, inbred P and NP rats were subjected to QTL analysis. A highly significant QTL segregating with alcohol preference on chromosome 4 (lod score 9.2) was observed. One gene from the genes detected by TOGA proved to be localized in that QTL on chromosome 4 -  $\alpha$ -synuclein (Liang et al., 2003). Synucleins have attracted much attention because of their involvement in several neurodegenerative disorders. In particular,  $\alpha$ -synuclein has been shown to be lethal to dopaminergic neurons in culture. An explanation of its special deleterious effect on dopaminergic neurons has been provided recently (Burke et al., 2003). They hypothesize that selective loss of dopaminergic neurons is due to toxicity of dopamine in particularly dopamine-rich brain regions like the substantia nigra. More than 50 publications can be found in the literature confirming the neurotoxicity of elevated amounts of dopamine (Fahn, 1997). Very likely, however, it is not dopamine itself but one of its metabolites, 3,4-dihydroxyphenyl acetaldehyde (DOPAL) that may be the culprit for the selective vulnerability of those types of neurons. The authors provide evidence that  $\alpha$ -synuclein becomes neurotoxic by interaction with DOPAL *in vivo*, which together generate free radicals, and conclude that selective vulnerability is due to the fact that only dopaminergic neurons synthesize dopamine and its metabolites in amounts sufficiently high to produce toxic levels of DOPAL. Whatever the mechanism might be of how  $\alpha$ -synuclein affects dopaminergic neurons, it is important to note that P rats seem to have an innate deficiency in the mesolimbic

dopaminergic pathway. Dopamine levels have been shown to be 30 % lower in the N. accumbens of P rats and it might well be that this neurochemical endophenotype contributes to enhanced alcohol preference. For a further discussion of expression profiling methods in combination with QTL analysis see Hoffman and Tabakoff (2005).

### **Beads Array for the Detection of Gene Expression (BADGE) and other bead-supported Techniques (Fiber optics, Microbead Libraries and MPSS)**

There has been a strong tendency to perform expression profiling on solid supports where molecular probes are immobilized. In this way, washing and postlabeling procedures are facilitated after hybridizations. Moreover, data collection by scanning is easily standardized. A variety of beads – magnetic, plastic, fluorescent – have been used to attach probes for profiling means. Due to their relatively little dimensions (50 nm–10  $\mu$ m), they allow for very small hybridization volumes (10–100  $\mu$ l). This feature may be central in experiments where only limited amounts of starting material are available. In order to achieve that goal, the beads have to be assembled at high density but, at the same time, be identified individually. The techniques developed by Luminex (Austin, Tx., USA) and Illumina (San Diego, Ca., USA) rely on a decoding system with multiple color combinations, i.e. “optical bar codes” (Yang et al., 2001). In the microbead libraries invented by Brenner et al. (2000 a,b), beads are sorted according to fluorescence intensities and hybridized cDNAs are sequenced *in situ*.

Another method employing large numbers of beads as probe carriers, as well, has been developed by Steemers et al. (2000). It is a self-assembled bead array immobilized on glass fiber tips. The ends of glass fibers can be etched to form wells, which can be filled with DNA-laden latex or silica beads. Thousands of such fibers can be physically bundled together. To ensure correct assignment of specific DNAs to the corresponding beads, the beads have to be encoded by unique combinations of fluorescent dyes, by an “optical bar code”, as already outlined above. Hybridization is carried out by just dipping the fiber ends into the hybridization solution.

In the meantime, the company pursuing this kind of approach (Illumina) has developed a combination of their beads with glass chips. Thousands of wells 20  $\mu$ m apart from each other are etched into the glass in a rectangular pattern and are filled with 3  $\mu$ m  $\varnothing$  beads. Each bead is loaded with gene-specific 50-mer oligonucleotides. There are always 2 distinct 50-mer probes per gene distributed on the beads with approximately 30-fold redundancy. The beads are randomly distributed into the wells and are decoded after the experiment to determine which probe sequence resides on the respective bead (see above). This allows for interrogating 12,000 transcripts per glass chip. Illumina maintains that chip-to-chip variability is less than 10 %, and typically less than 200 ng of total RNA starting material is sufficient for one hybridization. 1.3-fold changes of gene expression can reliably be measured. The chips are particularly suited to detect changes of low-abundant transcripts since the limit of detection at 99 % confidence lies in the range of 0.15 pM.

In general, typical features of small-sized three-dimensional structures, like beads, are their increasing surface areas

relative to decreasing volumes. As a consequence, many more probes can be assembled on beads compared to a similar-sized planar surface, and therefore, the concentration of target molecules on one bead can reach rather high levels (up to 1 mM). This phenomenon markedly enhances sensitivity and detection limits. The drawbacks of both the fiber optic and the other microbead approaches may reside in their complexities and requirements of expensive instrumentation.

### **Membrane-based Arrays**

Membrane-based arrays have been developed from the well-established NORTHERN blot hybridizations, that use electrophoretic separation prior to hybridization with radiolabeled probes. Alternatively, dot blots were used to circumvent electrophoretic separations and transfer to membranes. Macroarrays on nylon membranes are a consequent advancement thereof with spotted (unlabeled) probes and radiolabeled targets. Commercially available membranes can be obtained with several hundred or up to 1,176 selected probes (BD Biosciences-Clontech, Palo Alto, Ca., USA), with approximately 5,000 rat gene probes on GF300 filters (Research Genetics, Huntsville, AL, USA), or with up to 18,378 probes on gene discovery arrays (GDA membranes with spotted bacterial cDNA clones, Genome Systems Inc., St. Louis, MO, USA). Typically, membranes are hybridized with <sup>33</sup>P-labeled targets and hybrids are detected and quantified by a phosphorimager or autoradiogram. Since the know-how and pieces of equipment for the technique are available in every standard molecular biology laboratory, the method is easy to perform and inexpensive. Work with radiolabel makes it very sensitive but diminishes the resolution. To partially compensate for the latter, spots have to be rather far apart from each other, which requires large membranes, particularly with 18,000 probes or more.

Evidently, resolution can be improved by use of materials with more even surfaces where membrane deformations are abolished like glass or plastic. For those reasons, plastic slides (chips) have been developed that hold up to 12,000 oligonucleotide probes (BD-Clontech) and can be hybridized with radiolabel, as well.

Nylon (macro) arrays have the advantage of high sensitivity in general and of high fold-sensitivity. Many of the changes falling in the range below the 2-fold change threshold in fluorescence-based arrays can be reliably identified.

Nylon membranes have been used in a variety of studies in drug abuse research. Three investigations on cocaine effects on gene expression should be discussed here. Bibb et al. (2001) report on increases of the neuronal protein kinase cdk5, which is a target molecule downstream of  $\Delta$ fosB, both on the mRNA and protein level in caudate putamen and N. accumbens of mice, while PKA-dependent phosphorylation of dopamine and cyclic-AMP regulated phosphoprotein (DARPP-32) and expression of GluR1 subunit of AMPA-receptors were reduced upon chronic exposure to cocaine (Bibb et al., 2001). Roscovitine, an inhibitor of cdk5, normalized DARPP-32 phosphorylation and conductance of AMPA-receptors. In another study on monkeys, additional genes encoding intracellular signaling molecules have been identified (Freeman et al., 2001). Since profiling has also been performed in N. accumbens, it appears to be a good

complementation of the findings by Bibb et al. Apart from upregulated PKA, other kinases like cell adhesion tyrosine kinase  $\beta$  (PYK2) and MAPKK (MEK1) were upregulated. It is well known that PKA phosphorylates the transcription factor CREB, that has been shown previously to be induced by cocaine. PKA, however, also phosphorylates the AP1 proteins c-fos and junB. PYK2, on the other hand, phosphorylates MEK1, which can also phosphorylate AP1 proteins. Hence, cocaine elevates transcriptional levels of genes encoding enzymes of intracellular signaling cascades merging at the same endpoints. The other gene,  $\beta$ -catenin, that was upregulated upon cocaine administration, nicely fits in this view, since it reportedly influences jun gene transcription. More gene transcripts have been found regulated by chronic cocaine consumption in the report by Backes and Hemby (2003). In their self-administration paradigm, the animals were allowed free access to the drug for 1–20 days. The authors used laser capture microscopy (LCM) to excise tyrosine hydroxylase-positive neurons from the ventral tegmental area (VTA). 95 gene transcripts were array-screened and the most evident changes were found in the GABA<sub>A</sub> receptor subunit expression. With the exception of the  $\alpha_1$  subunit (upregulated), 5 other receptor subunits were down-regulated both at the beginning and end of drug exposure. The authors conclude, that impaired GABA<sub>A</sub> receptor function may facilitate dopamine release and transmission in N. accumbens. The third research report investigated expression changes occurring upon 3 weeks of cocaine withdrawal (Toda et al., 2002). The authors selected caudate putamen, prefrontal cortex and N. accumbens. The latter was subdivided into core and shell. Toda et al. have found BDNF-receptor (trkB) transcripts down-regulated in prefrontal cortex, whereas there was no change in N. accumbens core. However, BDNF-receptor protein was upregulated in N. accumbens core. This is a good example for the complexity of the brain. It nicely demonstrates that with increasing the number of brain regions included, transcriptional changes are becoming more and more ambiguous. Moreover, differential expression of mRNA and protein becomes equally important.

Expression profiling using nylon membrane arrays has also been performed in alcohol-treated mice and rats. Alcohol-preferring mice that were allowed free access to ethanol for 14 days showed increased transcriptions of I $\kappa$ B $\alpha$ , an activator of the transcription factor NF $\kappa$ B, and of clusterin which is an apolipoprotein (Murphy et al., 2002). More genes showed up in the study by Saito et al. (2002), who used arrays including more than 5,000 genes. Saito et al. used dorsal hippocampi from rats after 15 months of ethanol experience. Increased expression has been observed with genes related to ethanol-induced oxidative stress, like ceruloplasmin, uricase, p450-NAD<sup>+</sup> isocitrate dehydrogenase, or cytochrome C oxidase. The other group of genes markedly increased encoded gene products belonging to mediators of membrane-trafficking, like dynamin-1, dynein-associated polypeptide, phosphatidylinositol-4 kinase, RAB10, or ADP-ribosylation factor.

### Solid Support DNA-Chips

The majority of expression profiling experiments is presently being carried out on glass chips. Although a two-dimensional plane restricts miniaturization to some degree, read-out of

hybridization signals is facilitated. Typically, targets are labeled with fluorochromes that are detected by some fluorescence reader. It turns out, that glass surfaces have to be absolutely even and extremely clean to avoid serious artefacts. Therefore, chip production has to be done under clean-room conditions. Surface trimming and coating have to meet high standards of reproducibility. Standardized packing, shipping and storage conditions are further pivotal requirements for successful experiments.

### Affymetrix (Photolithography-Based Chips)

Affymetrix Inc. is one of the pioneers in chip technology. They have established a highly efficient but elaborate production process using photolithography to build up the probe oligonucleotides on the chip.

Due to the special production process, the length of probes cannot go much beyond 23–25 mer oligonucleotides. Probes cover a surface area of approximately 10  $\mu\text{m}^2$  and one chip can hold several hundred thousand spots. Due to the small spot size, a special scanner is needed for data collection. After completion of probe assembly, the chip is completely embedded in a dust-free metal housing and can only be accessed by two holes, through which hybridization, washing and staining solutions are provided. Each gene is represented by up to 20 distinct oligonucleotides. These 20 oligonucleotides are chosen from different regions of the mRNA (perfect match = PM) and in an additional, parallel row the same 20 nucleotides are deposited containing one mismatch, each (mismatch = MM). The target cRNAs from the cells or tissue under investigation are fractionated into small fragments before hybridization to ensure proper binding. Hybridization and washing conditions are such that perfect binding and mismatch binding should be clearly distinguishable. In this way, false positives can be excluded. Since only cRNAs from one sample are analyzed on one chip, correction for inter-chip variability becomes very important. Therefore, every chip contains a number of internal standards recognized by the specific Affymetrix software. The software package carries out data acquisition and storage in a format compatible with other programs that can perform further data processing, statistical analyses, clustering or data mining.

In the present issue, most of the publications using Affymetrix technology in respect to drug abuse research are reviewed. In particular, comprehensive views of genes affected by morphine and cocaine are provided by Ammon et al. (2003), Ammon-Treiber and Höllt (2005) and Yuferov et al. (2003; 2005). The paper by Yuferov et al. (2005) also summarizes studies carried out with amphetamine or methamphetamine using gene arrays. In the two most recent investigations on effects of these drugs in murine brain regions (Sokolov et al., 2003; Thomas et al., 2004), quite a number of genes have turned out to be differentially regulated. There is, however, little overlap, which is probably due to the different (but related) drugs or to the distinct brain regions analyzed. One gene upregulated in both studies is the transcription factor C/EBP. It also showed up in the reports by Freeman et al. (2001) and Tabakoff et al. (2003). Methamphetamine, likely by its neurotoxic influence on cortical neurons and on dopaminergic and serotonergic axon terminals in forebrain, appears to elicit relatively strong glial reactions, since genes

upregulated within 24h of treatment include glial fibrillary acidic protein (GFAP), an astrocytic marker, chemokines (CCL-12), cytokines (IL-1 $\alpha$ ), MHC class I, and the oncostatin receptor, which is part of the IL-6 receptor complex. Moreover, genes downregulated by methamphetamine in murine striatum can be grouped into the same category. Transthyretin, which can be increased by estrogens, has been described as a scavenger of amyloid  $\beta$  peptides and in this way likely lowers the risk of Alzheimer's disease (Tang et al., 2004). On the other hand, increased levels of transthyretin have been associated with depression-like behavior. It has been reported, that transthyretin-null mice exhibited increased exploratory activity (novelty seeking) (Sousa et al., 2004). Finally, the downregulation of the transcription factor early growth response (*egr*) may have some impact on normal circadian rhythms. It has been shown recently, that *egr-1* expression is induced in the medial preoptic, the periventricular (PVN), and in the suprachiasmatic nucleus (SCN) right at the beginning of the light phase (Davies et al., 2004).

Affymetrix chips have also been used to study transcriptional changes in response to ethanol. Rimondini et al. (2002) looked in prefrontal cortex and amygdala of rats exposed to alcohol. They found upregulated glutamate transporter (GLAST or EAAT1) and AMPA receptor subunit GluR-B in prefrontal cortex. Both biological responses can be interpreted as compensatory mechanisms to reduce enhanced activity of the glutamate system. Typically, GluR1, 3, and 4 AMPA receptor subunits ensure normal Ca<sup>2+</sup>-permeability. However, increased presence of GluR-2 gradually inhibits Ca<sup>2+</sup>-influx and, hence, prevents Ca<sup>2+</sup>-overload of the postsynaptic cell (Oguro et al., 2004; Petralia et al., 2004). Calnexin upregulation may be viewed in the same line. Calnexin appears to be involved in storage and release of intracellular calcium by its interaction with Ca<sup>2+</sup>-ATPase (Roderick et al., 2000). A comprehensive description of ethanol effects on expression patterns has been put together by Sommer et al. (2005).

In another study, Tabakoff et al. (2003) looked for differential gene expression in mice displaying high (HAFT) or low acute functional tolerance (LAFT) to alcohol. Amongst others, they found the GluR-1 subunit of AMPA receptors elevated in LAFT animals. Furthermore, Rad50 was increased. This protein plays a critical role in detection and repair of DNA double-strand breaks, in activation of cell cycle check points and in the maintenance of telomere integrity (Dasika et al., 1999). A more comprehensive coverage of these investigations has been provided by Hoffman and Tabakoff (2005).

Ethanol effects on gene expression have also been studied in cell cultures (Thibault et al., 2000). In human SH-SY5Y neuroblastoma cells, ethanol induced expression of noradrenaline-related genes, such as dopamine- $\beta$  hydroxylase and the sodium-dependent noradrenaline transporter. Consequently, increased noradrenaline release from these cells was observed. As already mentioned above, ethanol appears to impose stress on the cellular metabolism and, therefore, compensatory mechanisms to attenuate oxidative stress-related events have been found. In the cell cultures, transcription of glutathione-S-transferase and of additional enzymes involved in glutathione metabolism was increased. A more detailed review on the use of microarrays in cell culture models of drug effects has been provided elsewhere (Thibault et al., 2005).

Despite the high precision and quality of data obtained from Affymetrix chip experiments, it has to be kept in mind that the technology is expensive – very expensive. Detailed studies such as time-courses, concentration dependencies, investigations of specific brain regions in a larger number of animals are soon out of reach of financial resources. Even for well-funded medium-sized research institutions, it is often beyond their financial capacities to rely only on the Affymetrix technology.

*cDNA Chips* For those and a number of other reasons, many laboratories worldwide have established DNA chip facilities using commercially available chips or making their own, home-made chips. Pioneering work in this respect has come from Patrick O. Brown's laboratory at Stanford University (for review see: Brown and Botstein, 1999). This strategy allows for more flexibility and is less cost-intensive (Duggan et al., 1999). There is no doubt, that expensive pieces of technical equipment, such as pipetting robots, chip spotting devices, hybridization stations, and chip scanners are required to carry out the experiments. They are, however, less expensive than the Affymetrix setup. Moreover, due to distinct ways of production, the chips themselves are much cheaper. Typically, probe cDNAs are attached to glass slides by UV-crosslinking or covalent chemical bonding and hybridized to fluorescent target cDNAs. Hybridized slides are scanned in double-beam readers and fluorescence intensities are correlated to up- or downregulated gene transcripts.

To attach DNAs, glass surfaces can be chemically modified by introducing reactive groups such as aldehyde, epoxy or other residues, or can be coated with collagen or poly-lysine. Coating can be done in the laboratory or slides ready to use are also commercially available. Prices range between US \$ 5–15. Sources of DNAs to be spotted on the slides can be inserts from bacterial clones, PCR-products, or oligonucleotides. Oligonucleotides with sizes between 50 and 70 nt appear to be most suitable for this kind of chips. An amide-modified 3'-end provides the chemistry required for covalent attachment to the glass surface. When PCR-products are used, NH-modification of one primer is recommended, as well. Many people use bacterial clones as starting material. Clones can be obtained by purchasing custom-made, normalized libraries, or by buying single clones selected from well-characterized collections of clones (e.g. from Incyte Genomics Inc., Palo Alto, Ca., or RZPD, Berlin). Bacterial aliquots are added to PCR-mixtures and plasmid inserts are amplified using a plasmid-specific pair of primers annealing as closely as possible to the insert. PCR-products are either purified on Qiagen- or Millipore-multiwell plates or PCR-mixtures are used directly for spotting (Diehl et al., 2002).

Quite a number of spotting techniques have been developed including ink-jet techniques as used in printers. The standard procedure is still using either solid or split pins. Split pins are equipped with a reservoir enabling multiple spotting without retrieving DNA solution from the master plate every time. In this way, high-density arrays of up to 30,000 DNA-spots can be made on one chip. Typically, spot diameters are in the range of 100–150  $\mu$ m, but much smaller spots have been obtained using more hydrophobic glass surfaces or modifying the composition of spotting solutions.

Hybridizations to the probe DNAs have been carried out with fluorescence-labeled cDNAs or cRNAs. There is a

variety of labeling options including one-step labeling with cyanine-bound nucleotides or incorporation of an amino-allyl-modified nucleotide in the reverse transcription reaction and a post-labeling with fluorochrome. Often, chips are hybridized with cDNAs/cRNAs derived from control and treated samples, simultaneously. In this case, the two nucleic acid populations carry two different fluorescence labels. All fluorescence readers commercially available for chip scanning are able to read at least two different fluorochromes, some can even read up to four. There are many ways of generating labeled target cDNAs/cRNAs and standardization has become a major issue. If RNA preparations, reverse transcriptions and cDNA labelings are not subject to internationally accepted quality criteria, chip results obtained in one laboratory cannot be compared with results from another one. These issues have been covered by reviews of Reimers (2005) and Soverchia et al. (2005).

Chip hybridizations have been carried out in various ways, too. At the beginning – and still used – is the cover glass method. Its clear advantage is its ease of use, low volume of hybridization solution, and negligible cost. Some drawbacks reside in its tendency to create high background staining at the edges where evaporation is high. People have tried to compensate for this by putting the slides in tightly sealed humid chambers. The low hybridization volume can cause problems, as well, because mixing is extremely slow. To avoid this phenomenon, hybridization volume has been increased by putting posts under the four corners of the cover slip. An option has been to glue a disposable chamber on the chip (Grace Bio-Labs, Bend, Or.). The chamber is sealed by a transparent plastic cover and filled through an inlet on one side of the chamber. After filling, both inlet and outlet holes are taped and the whole setup can be put in the appropriate conditions, including shaking on a shaker. More sophisticated hybridization chambers have been developed by Ambion, Austin, Tx., Genetix, New Milton, Hampshire, U.K., Telechem, Sunnyvale, Ca., Genemachines, San Carlos, Ca., Monterey Ind., Richmond, Ca., Schleicher & Schuell, Keene, N.H., Tecan, Dorset, U.K., Thermo Hybaid, Woburn, Ma.. For more detailed descriptions of how to produce chips and how to process expression data after scanning the chips, the reader is directed to the reviews by Bowtell (1999), Cheung et al. (1999), and Barr and Gao (2005).

At this point, it appears appropriate to summarize pros and cons of the two chip technologies in a short overview (Table 1).

Numerous expression profiling studies have been carried out using cDNA chips. In drug abuse research, effects of cocaine consumption on expression patterns have been studied in brains of dopamine D1 and D3 receptor mutant mice following acute and chronic treatments (Zhang et al., 2002; 2004). In the first study by Zhang et al. (2002), 54 genes were found to be differentially regulated between wild-type and D1 receptor-deficient mice. In their follow-up investigation, where they used Affymetrix chips, they included the D3 receptor mutants in their experiments. Since both types of receptors are expressed in the same neurons, in N. accumbens and in caudate putamen, in particular, the mutants have been used to reveal the specific effects of each receptor separately on gene expression in the presence of cocaine. The data strongly suggest that transcripts of signaling molecules like ERK1 or c-fos are induced by D1 receptor but inhibited by D3 receptor activation in CPU. Moreover, molecules possessing consensus sequences for CREB or AP1, like dynorphin, neogenin, or synaptotagmin VII were regulated in the same way as ERK1 and c-fos.

In other studies, cDNA technology has been used to investigate expression profiles characteristic for alcohol abuse (Mayfield et al., 2002). Material from human frontal and motor cortex has been chosen to interrogate 5600–7200 gene targets. 191 of them showed differential expression values of greater than 1.4-fold, the majority of which were down-regulated in the group of alcoholics. Apart from genes previously described as alcohol-responsive, like apolipoprotein A-I, neuropeptide Y, or thyroid hormone receptor  $\beta\epsilon$  it turned out that expression of genes involved in myelination was consistently affected by ethanol. This finding highlights again the close involvement of glial cells in alcohol-dependent brain injuries. Myelin-associated oligodendrocyte basic protein (MOG) was upregulated both in frontal and motor cortex, whereas the astrocytic protein GFAP was downregulated in frontal cortex. Phospholipase A2, an enzyme also involved in lipid degradation was upregulated in both brain regions, as well. Furthermore, there were a number of synapse-associated proteins, like syntaxin binding protein, synapsin II, and vesicle-associated membrane protein (VAMP) 3 being down-regulated by ethanol. Finally, some important transcripts encoding intracellular signaling molecules, like CREM, PKA type-I $\alpha$ , or cellular retinoic acid-binding proteins 1 and 2, were downregulated, as well. In a subsequent study by the same group, a few additional interesting genes showed up (Liu

Table 1.

<i>Affymetrix GeneChip</i>	<i>Spotted MicroArray</i>
25-mer oligonucleotide probe pair sets	Oligonucleotide (50–70-mers) or cDNA clones
Synthesized <i>in situ</i>	Physically placed on solid support
Very high probe density	High probe density
Requires sequence information	Sequence information not necessarily required
Complex chip design	Less complex array design
No infrastructure needed	Clone management needed
Commercially available	Commercial and ‘Do it Yourself’ approaches
Limited ability to customize	Custom Arrays
Very expensive per array	Inexpensive to moderately expensive per array
Single signal detection	Dual (two-color) signal detection
Complete system available	Components available

et al., 2004). One gene upregulated in frontal cortex but down-regulated in motor cortex was neutral sphingomyelinase. Neutral sphingomyelinase generates ceramides that trigger intracellular signaling cascades leading to apoptotic cell death, i.e. ceramide formation, c-Jun N-terminal kinase phosphorylation, caspase-3 activation, and DNA fragmentation in the nuclei. This would be a good explanation for the brain damage observed in frontal cortex of alcoholics (Kril and Halliday, 1999; Lewohl et al., 2000; 2001). Consequently, specific inhibition of this enzyme could prevent neuronal loss in this brain region (Soeda et al., 2004). The single-stranded DNA- and RNA-binding protein, purine-rich element binding protein  $\alpha$  (Pur- $\alpha$ ), has been found upregulated in motor cortex of alcoholics. It is implicated in many biological processes, including control of transcription of multiple genes, initiation of DNA replication, and RNA transport and translation. Mice with targeted disruption of the PURA gene in both alleles appear normal at birth. However, at 2 weeks of age, they develop severe tremor and spontaneous seizures, and they die by 4 weeks. The numbers of neurons in hippocampus and cerebellum of these animals are markedly lower than those of their age-matched wild-type littermates, and lamination of these regions is aberrant at time of death (Khalili et al., 2003). Hence, PURA upregulation in alcoholics may be an important protective mechanism in motor cortex.

### Concluding remarks and summary

Techniques of expression profiling, as described here, have been used very successfully in many research applications, preferentially in lower organisms, in cell culture systems, or in cancer research. It has turned out very clearly that the homogeneity of the starting material is crucial for the interpretation of results. Even in "simple" mammalian systems, i.e. in solid tumors where malignant and non-malignant cells are in close neighborhood, reliable data about transcriptional changes in the malignant cells could only be obtained by laser capture dissection of this cell type. Expression profiling of all cells of the tumor largely obscured the changes specific for the tumor cells. This problem arises for all kinds of *ex vivo* material, the more so in studies using brain tissue. Upon viewing the data from expression profilings referred to in this paper, there is hardly any overlap of genes induced or repressed in independent studies even when the same drug had been used. There are long lists of genes differentially expressed upon exposure to ethanol. Most of them could be expressed in any cell type of the brain, and only few of them can be specifically associated with certain types of neurons. These, however, have often already been studied previously in "single gene" approaches, so the microarray data serve only to confirm what was anticipated. Moreover, one might be running into similar problems as before when, for example, it is not a receptor that is upregulated, but only one subunit, whereas the other subunits are downregulated. And it may happen vice versa in another brain region. This example shows that heterogeneity does not stop at the level of single cells, but needs to be taken into consideration at the molecular level, as well.

The review is not meant to describe all techniques of expression profiling being available, but tries to focus on those that have been used in studies on drug abuse research.

Additional ones not (yet) used in this specific field and, hence, only briefly described here, have been selected with respect to their technically demanding features and smart alternative solutions to perform expression profiling. The review is intended to encourage researchers to use these techniques more often in the awareness that the techniques are already well advanced. It is no major problem to assemble any number of molecular probes very reliably in any kind of array system and to carry out reproducible hybridizations. It should be kept in mind, however, that the bulk of noise is added to the system by the starting material and its preparation for the profiling. Even the cleanest chip experiment can be to no avail if the starting material has not been selected carefully and processed reproducibly enough.

The intriguing feature of expression profilings is their multiplex character. The large numbers of specific reactions occurring simultaneously in solution or on chips provide researchers with an as yet unrivaled performance of experiments and immense time savings. The technologies abandon the basic principle of most research strategies that proceed in step by step fashions. Iterative procedures have been the research philosophy of choice to keep experimental progress under control. They have also been the fundamental philosophy of computer technology. By and large, both are going to be replaced by systems working in a highly parallel fashion. In essence, people have learned from processes naturally occurring in living organisms. Without specific, highly coordinated, and simultaneously running reactions, biological systems would not be existent. Nature with its extremely efficient organisational structures is our best teacher.

### Acknowledgements

Supported by DFG grant Ge 486/11-4 and by EC-grant TARGALC QLG3-CT-2002-01048.

### References

- Ammon S, Mayer P, Riechert U, Tischmeyer H, Hollt V (2003) Microarray analysis of genes expressed in the frontal cortex of rats chronically treated with morphine and after naloxone precipitated withdrawal. *Brain Res Mol Brain Res* 112:113–125.
- Ammon-Treiber S, Höllt V (2005) Morphine-induced Changes of Gene Expression in the Brain. *Addiction Biol* 10:81–89.
- Anderson JL, Head SI, Morley JW (2003) Altered inhibitory input to Purkinje cells of dystrophin-deficient mice. *Brain Res* 982:280–283.
- Backes E, Hemby SE (2003) Discrete cell gene profiling of ventral tegmental dopamine neurons after acute and chronic cocaine self-administration. *J Pharmacol Exp Ther* 307:450–459.
- Bahi A, Boyer F, Kafri T, Dreyer JL (2004) CD81-induced behavioural changes during chronic cocaine administration: in vivo gene delivery with regulatable lentivirus. *Eur J Neurosci* 19:1621–1633.
- Barr GA, Gao P (2005) Issues for consideration in the analysis of microarray data in behavioral studies. *Add Biol* 10:15–21.
- Bibb JA, Chen J, Taylor JR, Svenningsson P, Nishi A, Snyder GL, Yan Z, Sagawa ZK, Ouimet CC, Nairn AC, Nestler EJ, Greengard P (2001) Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature* 410:376–380.
- Bowtell DD (1999) Options available—from start to finish—for obtaining expression data by microarray. *Nat Genet* 21:25–32.
- Brenner S, Williams SR, Vermaas EH, Storck T, Moon K, McCollum C, Mao JI, Luo S, Kirchner JJ, Eletr S, DuBridge RB, Burcham T, Albrecht G (2000a) In vitro cloning of complex mixtures of DNA on microbeads: physical separation of differentially expressed cDNAs. *Proc Natl Acad Sci USA* 97:1665–1670.

- Brenner S, Johnson M, Bridgham J, Golda G, Lloyd DH, Johnson D, Luo S, McCurdy S, Foy M, Ewan M, Roth R, George D, Eletr S, Albrecht G, Vermaas E, Williams SR, Moon K, Burcham T, Pallas M, DuBridge RB, Kirchner J, Fearon K, Mao J, Corcoran K (2000b) Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nat Biotechnol* 18:630–634.
- Brenz Verca MS, Widmer DA, Wagner GC, Dreyer J (2001) Cocaine-induced expression of the tetraspanin CD81 and its relation to hypothalamic function. *Mol Cell Neurosci* 17:303–316.
- Brown PO, Botstein D (1999) Exploring the new world of the genome with DNA microarrays. *Nat Genet* 21:33–37.
- Burke WJ, Li SW, Williams EA, Nonneman R, Zahm DS (2003) 3,4-Dihydroxyphenylacetaldehyde is the toxic dopamine metabolite in vivo: implications for Parkinson's disease pathogenesis. *Brain Res* 989:205–213.
- Camargo AA, Samaia HPB, Dias-Neto E, Simão DF, Migotto IA, Briones MRS, Costa FF, Nagai MA, Verjovski-Almeida S, Zago MA, Andrade LEC, Carrer H, El-Dorry HFA, Esprefico EM, Habr-Gama A, Giannella-Neto D, Goldman GH, Gruber A, Hackel C, Kimura ET, Maciel RMB, Marie SKN, Martins EAL, Nóbrega MP, Paçó-Larson ML, Pardini MIMC, Pereira GG, Pesquero JB, Rodrigues V, Rogatto SR, da Silva IDCG, Sogayar MC, Sonati MF, Tajara EH, Valentini SR, Alberto FL, Amaral MEJ, Aneas I, Arnaldi LAT, de Assis AM, Bengtson MH, Bergamo NA, Bombonato V, de Camargo MER, Canevari RA, Carraro DM, Cerutti JM, Corrêa MLC, Corrêa RFR, Costa MCR, Curcio C, Hokama POM, Ferreira AJS, Furuzawa GK, Gushiken T, Ho PL, Kimura E, Krieger JE, Leite LCC, Majumder P, Marins M, Marques ER, Melo ASA, Barbosa de Melo M, Mestriner CA, Miracca EC, Miranda DC, Nascimento ALTO, Nóbrega FG, Ojopi ÉPB, Pandolfi JRC, Pessoa LG, Prevedel AC, Rahal P, Rainho CA, Reis EMR, Ribeiro ML, da Rós N, de Sá RG, Sales MM, Sant'anna SC, dos Santos ML, da Silva AM, da Silva NP, Silva WA, da Silveira RA Jr, Sousa JF, Stecconi D, Tsukumo F, Valente V, Soares F, Moreira ES, Nunes DN, Correa RG, Zalberg H, Carvalho AF, Reis LFL, Brentani RR, Simpson AJG, de Souza SJ (2001) The contribution of 700,000 ORF sequence tags to the definition of the human transcriptome. *Proc Natl Acad Sci USA* 98:12103–12108.
- Cheung VG, Morley M, Aguilar F, Massimi A, Kucherlapati R, Childs G (1999) Making and reading microarrays. *Nat Genet* 21:15–19.
- Dasika GK, Lin SC, Zhao S, Sung P, Tomkinson A, Lee EY (1999) DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis. *Oncogene* 18:7883–7899.
- Davies JS, Carter DA, Wells T (2004) Photic stimulation inhibits growth hormone secretion in rats: a hypothalamic mechanism for transient entrainment. *Endocrinology* 145:2950–2958.
- Dias-Neto E, Correa RG, Almeida SV, Briones MRS, Nagai MA, da Silva W Jr, Zago MA, Bordin S, Costa FF, Goldman GH, Carvalho AF, Matsukuma A, Baia GS, Simpson DH, Brunstein A, de Oliveira PSL, Bucher P, Jongeneel VC, O'Hare MJ, Soares F, Brentani RR, Reis LFL, de Souza SJ, Simpson AJG (2000) Shotgun sequencing of the human transcriptome with ORF expressed sequence tags. *Proc Natl Acad Sci USA* 97:3491–3496.
- Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci USA* 93:6025–6030.
- Diehl F, Beckmann B, Kellner N, Hauser NC, Diehl S, Hoheisel JD (2002) Manufacturing DNA microarrays from unpurified PCR products. *Nucleic Acids Res* 30:e79.
- Duggan DJ, Bittner M, Chen Y, Meltzer P, Trent JM (1999) Expression profiling using cDNA microarrays. *Nat Genet* 21:10–14.
- Evans SJ, Datson NA, Kabbaj M, Thompson RC, Vreugdenhil E, De Kloet ER, Watson SJ, Akil H (2002) Evaluation of Affymetrix Gene Chip sensitivity in rat hippocampal tissue using SAGE analysis. *Serial Analysis of Gene Expression. Eur J Neurosci* 16:409–413.
- Fahn S (1997) Levodopa-induced neurotoxicity. *CNS Drugs* 8:376–393.
- Fan L, van der Brug M, Chen W, Dodd PR, Matsumoto I, Niwa S, Wilce PA (1999) Increased expression of mitochondrial genes in human alcoholic brain revealed by differential display. *Alcohol Clin Exp Res* 23:408–413.
- Follesa P, Ticku MK (1995) Chronic ethanol treatment differentially regulates NMDA receptor subunit mRNA expression in rat brain. *Brain Res Mol Brain Res* 29:99–106.
- Freeman WM, Nader MA, Nader SH, Robertson DJ, Gioia L, Mitchell SM, Daunais JB, Porrino LJ, Friedman DP, Vrana KE (2001) Chronic cocaine-mediated changes in non-human primate nucleus accumbens gene expression. *J Neurochem* 77:542–549.
- Hoffman PL, Tabakoff B (2005) Gene expression in animals with different acute responses to ethanol. *Addiction Biol* 10:91–100.
- Ito T, Hozumi Y, Sakane F, Saino-Saito S, Kanoh H, Aoyagi M, Kondo H, Goto K (2004) Cloning and characterization of diacylglycerol kinase iota splice variants in rat brain. *J Biol Chem* 279:23317–23326.
- Jacobs EH, Spijker S, Verhoog CW, Kamprath K, de Vries TJ, Smit AB, Schoffelmeer AN (2002) Active heroin administration induces specific genomic responses in the nucleus accumbens shell. *FASEB J* 16:1961–1963.
- Jacobs EH, Smit AB, de Vries TJ, Schoffelmeer ANM (2005) Long-term gene expression in the nucleus accumbens following heroin administration is subregion-specific and depends on the nature of drug administration. *Addiction Biol* 10:91–100.
- Khalili K, Del Valle L, Muralidharan V, Gault WJ, Darbinian N, Otte J, Meier E, Johnson EM, Daniel DC, Kinoshita Y, Amini S, Gordon J (2003) Puralpha is essential for postnatal brain development and developmentally coupled cellular proliferation as revealed by genetic inactivation in the mouse. *Mol Cell Biol* 23:6857–6875.
- Kril JJ, Halliday GM (1999) Brain shrinkage in alcoholics: a decade on and what have we learned? *Prog Neurobiol* 58:381–387.
- Lewohl JM, Wang L, Miles MF, Zhang L, Dodd PR, Harris RA (2000) Gene expression in human alcoholism: microarray analysis of frontal cortex. *Alcohol Clin Exp Res* 24:1873–1882.
- Lewohl JM, Dodd PR, Mayfield RD, Harris RA (2001) Application of DNA microarrays to study human alcoholism. *J Biomed Sci* 8:28–36.
- Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967–971.
- Liang T, Spence J, Liu L, Strother WN, Chang HW, Ellison JA, Lumeng L, Li TK, Foroud T, Carr LG (2003) alpha-Synuclein maps to a quantitative trait locus for alcohol preference and is differentially expressed in alcohol-preferring and -nonpreferring rats. *Proc Natl Acad Sci USA* 100:4690–4695.
- Liu J, Lewohl JM, Dodd PR, Randall PK, Harris RA, Mayfield RD (2004) Gene expression profiling of individual cases reveals consistent transcriptional changes in alcoholic human brain. *J Neurochem* 90:1050–1058.
- Mayfield RD, Lewohl JM, Dodd PR, Herlihy A, Liu J, Harris RA (2002) Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics. *J Neurochem* 81:802–813.
- Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR (1984) Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res* 12:7035–7056.
- Menssen A, Hermeking H (2002) Characterization of the c-MYC-regulated transcriptome by SAGE: identification and analysis of c-MYC target genes. *Proc Natl Acad Sci USA* 99:6274–6279.
- Michna L, Brenz Verca MS, Widmer DA, Chen S, Lee J, Rogove J, Zhou R, Tsitsikov E, Miescher GC, Dreyer JL, Wagner GC (2001) Altered sensitivity of CD81-deficient mice to neurobehavioral effects of cocaine. *Brain Res Mol Brain Res* 90:68–74.
- Murphy BC, Chiu T, Harrison M, Uddin RK, Singh SM (2002) Examination of ethanol responsive liver and brain specific gene expression, in the mouse strains with variable ethanol preferences, using cDNA expression arrays. *Biochem Genet* 40:395–410.

- Oguro K, Miyawaki T, Yokota H, Kato K, Kamiya T, Katayama Y, Fukaya M, Watanabe M, Shimazaki K (2004) Upregulation of GluR2 decreases intracellular Ca<sup>2+</sup> following ischemia in developing gerbils. *Neurosci Lett* 364:101–105.
- Peng W, Simantov R (2003) Altered gene expression in frontal cortex and midbrain of 3,4-methylenedioxy-methamphetamine (MDMA) treated mice: differential regulation of GABA transporter subtypes. *J Neurosci Res* 72:250–258.
- Petralia RS, Sans N, Wang YX, Vissel B, Chang K, Noben-Trauth K, Heinemann SF, Wenthold RJ (2004) Loss of GLUR2 alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit differentially affects remaining synaptic glutamate receptors in cerebellum and cochlear nuclei. *Eur J Neurosci* 19:2017–2029.
- Reimers M (2005) Statistical Analysis of Microarray Data. *Add Biol* 10.
- Ren LQ, Gourmal N, Boddeke HW, Gebicke-Haerter PJ (1998) Lipopolysaccharide-induced expression of IP-10 mRNA in rat brain and in cultured rat astrocytes and microglia. *Brain Res Mol Brain Res* 59:256–263.
- Rimondini R, Arlinde C, Sommer W, Heilig M (2002) Long-lasting increase in voluntary ethanol consumption and transcriptional regulation in the rat brain after intermittent exposure to alcohol. *FASEB J* 16:27–35.
- Roderick HL, Lechleiter JD, Camacho P (2000) Cytosolic phosphorylation of calnexin controls intracellular Ca<sup>2+</sup> oscillations via an interaction with SERCA2b. *J Cell Biol* 149:1235–1248.
- Saito M, Smiley J, Toth R, Vadasz C (2002) Microarray analysis of gene expression in rat hippocampus after chronic ethanol treatment. *Neurochem Res* 27:1221–1229.
- Soeda S, Tsuji Y, Ochiai T, Mishima K, Iwasaki K, Fujiwara M, Yokomatsu T, Murano T, Shibuya S, Shimeno H (2004) Inhibition of sphingomyelinase activity helps to prevent neuron death caused by ischemic stress. *Neurochem Int* 45:619–626.
- Sogos V, Reali C, Fanni V, Curto M, Gremo F (2003) Dystrophin antisense oligonucleotides decrease expression of nNOS in human neurons. *Brain Res Mol Brain Res* 118:52–59.
- Sommer W, Arlinde C, Caberlotto L, Thorsell A, Hyytia P, Heilig M (2001) Differential expression of diacylglycerol kinase iota and L18A mRNAs in the brains of alcohol-preferring AA and alcohol-avoiding ANA rats. *Mol Psychiatry* 6:103–108.
- Sommer W, Arlinde C, Heilig M (2005) The Search for Candidate Genes of Alcoholism: Evidence from Expression Profiling Studies. *Add Biol* 10:71–79.
- Sokolov BP, Poleskaya OO, Uhl GR (2003) Mouse brain gene expression changes after acute and chronic amphetamine. *J Neurochem* 84:244–252.
- Sousa JC, Grandela C, Fernandez-Ruiz J, de Miguel R, de Sousa L, Magalhaes AI, Saraiva MJ, Sousa N, Palha JA (2004) Transthyretin is involved in depression-like behaviour and exploratory activity. *J Neurochem* 88:1052–1058.
- Soverchia L, Leonardi-Essmann F, Ubaldi M, Ciccocioppo R, Hardiman G (2005) Microarrays and the interrogation of brain gene expression – The challenge of sample preparation. *Add Biol* 10:5–13.
- Spence JP, Liang T, Foroud T, Lo D, Carr LG (2005) Expression Profiling And QTL Analysis: A Powerful Complementary Strategy In Drug Abuse Research. *Add Biol* 10:47–51.
- Stemmers FJ, Ferguson JA, Walt DR (2000) Screening unlabeled DNA targets with randomly ordered fiber-optic gene arrays. *Nat Biotechnol* 18:91–94.
- Surka MC, Tsang CW, Trimble WS (2002) The mammalian septin MSF localizes with microtubules and is required for completion of cytokinesis. *Mol Biol Cell* 13:3532–3545.
- Sutcliffe JG, Foye PE, Erlander MG, Hilbush BS, Bodzin LJ, Durham JT, Hasel KW (2000) TOGA: an automated parsing technology for analyzing expression of nearly all genes. *Proc Natl Acad Sci USA* 97:1976–1981.
- Tabakoff B, Bhavé SV, Hoffman PL (2003) Selective breeding, quantitative trait locus analysis, and gene arrays identify candidate genes for complex drug-related behaviors. *J Neurosci* 23:4491–4498.
- Tang YP, Haslam SZ, Conrad SE, Sisk CL (2004) Estrogen increases brain expression of the mRNA encoding transthyretin, an amyloid beta scavenger protein. *J Alzheimers Dis* 6:413–420.
- Thibault C, Lai C, Wilke N, Duong B, Olive MF, Rahman S, Dong H, Hodge CW, Lockhart DJ, Miles MF (2000) Expression profiling of neural cells reveals specific patterns of ethanol-responsive gene expression. *Mol Pharmacol* 58:1593–1600.
- Thibault C, Hassan S, Miles MF (2005) Using in vitro models for expression profiling studies on ethanol and drugs of abuse. Molecular signature of drugs of abuse in cell culture. *Add Biol* 10:23–62.
- Thomas DM, Francescutti-Verbeem DM, Liu X, Kuhn DM (2004) Identification of differentially regulated transcripts in mouse striatum following methamphetamine treatment - an oligonucleotide microarray approach. *J Neurochem* 88:380–393.
- Toda S, McGinty JF, Kalivas PW (2002) Repeated cocaine administration alters the expression of genes in corticolimbic circuitry after a 3-week withdrawal: a DNA macroarray study. *J Neurochem* 82:1290–1299.
- Vega IE, Hsu SC (2003) The septin protein Nedd5 associates with both the exocyst complex and microtubules and disruption of its GTPase activity promotes aberrant neurite sprouting in PC12 cells. *Neuroreport* 14:31–37.
- Velculescu VE, Zhang L, Vogelstein B, Kinzler KW (1995) Serial analysis of gene expression. *Science* 270:484–487.
- Velculescu VE, Zhang L, Zhou W, Vogelstein J, Basrai MA, Bassett DE Jr, Hieter P, Vogelstein B, Kinzler KW (1997) Characterization of the yeast transcriptome. *Cell* 88:243–251.
- Vikolinsky R, Siggins GR, Campbell IL, Krucker T (2004) Acute exposure to CXC chemokine ligand 10, but not its chronic astroglial production, alters synaptic plasticity in mouse hippocampal slices. *J Neuroimmunol* 150:37–47.
- Wallis T, Bubbb WA, McQuillan JA, Balcar VJ, Rae C (2004) For want of a nail. Ramifications of a single gene deletion, dystrophin, in the brain of the mouse. *Front Biosci* 9:74–84.
- Yang L, Tran DK, Wang X (2001) BADGE, Beads Array for the Detection of Gene Expression, a high-throughput diagnostic bioassay. *Genome Res* 11:1888–1898.
- Yang J, Zoeller RT (2002) Differential display identifies neuroendocrine-specific protein-A (NSP-A) and interferon-inducible protein 10 (IP-10) as ethanol-responsive genes in the fetal rat brain. *Brain Res Dev Brain Res* 138:117–133.
- Yuferov V, Krosiak T, Laforge KS, Zhou Y, Ho A, Kreek MJ (2003) Differential gene expression in the rat caudate putamen after “binge” cocaine administration: advantage of triplicate microarray analysis. *Synapse* 48:157–169.
- Yuferov V, Nielsen DA, Butelman ER, Kreek MJ (2005) Microarray studies of psychostimulant-induced changes in gene expression. *Add Biol* 10:101–118.
- Zhang J, Zhang D, Xu M (2002) Identification of chronic cocaine-induced gene expression through dopamine d1 receptors by using cDNA microarrays. *Ann N Y Acad Sci* 965:1–9.
- Zhang L, Lou D, Jiao H, Zhang D, Wang X, Xia Y, Zhang J, Xu M (2004) Cocaine-induced intracellular signaling and gene expression are oppositely regulated by the dopamine D1 and D3 receptors. *J Neurosci* 24:3344–3354.
- Zhou Y, Spangler R, Schlussman SD, Yuferov VP, Sora I, Ho A, Uhl GR, Kreek MJ (2002) Effects of acute “binge” cocaine on preprodynorphin, preproenkephalin, proopiomelanocortin, and corticotropin-releasing hormone receptor mRNA levels in the striatum and hypothalamic-pituitary-adrenal axis of mu-opioid receptor knockout mice. *Synapse* 45:220–229.