Alcohol intake at levels posing an acute health risk is common amongst teenagers. Alcohol abuse is the second most common mental disorder worldwide. The incidence of smoking is decreasing in the Western world but increasing in developing countries and is the leading cause of preventable death worldwide. Considering the longstanding history of alcohol and tobacco consumption in human societies, it might be surprising that the molecular mechanisms underlying alcohol and smoking dependence are still incompletely understood. Effective treatments against the risk of relapse are lacking. Drugs of abuse exert their effect manipulating the dopaminergic mesocorticolimbic system. In this brain region, alcohol has many potential targets including membranes and several ion channels, while other drugs, for example nicotine, act via specific receptors or binding proteins. Repeated consumption of drugs of abuse mediates adaptive changes within this region, resulting in addiction. The high incidence of alcohol and nicotine co-abuse complicates analysis of the molecular basis of the disease. Gene expression profiling is a useful approach to explore novel drug targets in the brain. Several groups have utilised this technology to reveal drug-sensitive pathways in the mesocorticolimbic system of animal models and in human subjects. These studies are the focus of the present review.
if not all, drugs of abuse. Dopaminergic neural projections arise in the ventral tegmental area (VTA) in the midbrain and project to several forebrain regions including the nucleus accumbens (NAC) and the prefrontal cortex (PFC). Addictive drugs increase extracellular dopamine preferentially in the NAC either directly or via action on the VTA\(^{(13,14)}\). This is significant because the NAC plays a crucial role in learned reward anticipation\(^{(15)}\), drug-related learning\(^{(16)}\) and craving\(^{(17)}\). Repeated abuse results in tolerance to the drug’s effects and ultimately results in dependence which is characterised by withdrawal symptoms upon cessation of drug use\(^{(18,19)}\). Craving may persist for months or years after cessation and is likely to be a consequence of changes in cell structure, function or connectivity induced by long-term drug exposure.

Alcohol’s action on the MDS is complex. Alcohol does not bind a specific receptor; instead, it interferes with the activity of a number of proteins including ligand-gated ion channels\(^{(20)}\). In animals, acute alcohol enhances \(\gamma\)-aminobutyric acid (GABA)ergic and inhibits glutamatergic neurotransmission within the MDS\(^{(20)}\). Prolonged drinking has the opposite effect, resulting in region-specific alteration of GABA and glutamate receptor subtype expression\(^{(20)}\). Nicotine enhances an excitatory, glutamatergic drive on dopaminergic neurons by binding to specific, pre- and postsynaptic nicotinic acetylcholine receptors in the VTA\(^{(21-23)}\).

The present review focuses on alcohol and nicotine abuse and gives an insight into current approaches that reveal the molecular networks underlying dependence on these drugs. Microarray gene expression profiling simultaneously compares the expression levels of thousands of genes in a tissue. Linear amplification of total RNA allows the use of small amounts of starting material and is a useful tool for exploratory investigation of gene expression in tissue with limited availability. These technologies can provide a snapshot of the transcriptome of specific brain regions relevant to drug addiction. As reviewed below, most work to date has been conducted in animal models. Additionally, several research groups have embarked on elucidating the impact of drugs of abuse on the transcriptome of the human brain. In parallel studies, several groups have characterised the protein changes in selected brain regions of the alcoholic using high-throughput proteomics.

**Alcohol-related brain gene expression in animal models**

The heritability of alcoholism is estimated to be as high as 50 to 60\%\(^{(24,25)}\). Most diagnostic criteria of alcoholism are behavioural, including failure to control drinking quantity and frequency and continuation with knowledge of the social and medical consequences. It has been argued that there is no rodent model that resembles human alcoholism in all important aspects\(^{(26)}\). However, the power of rodent genetics allows a focus on the genetic basis of specific behavioural traits\(^{(26)}\). Several rodent strains have been selected for display of particular alcohol-related behaviours and then used to identify candidate genes associated with these behaviours. To identify genetic components, two inbred strains, which differ in the response of interest, are chosen and intercrossed to produce recombinant inbred strains\(^{(27)}\). The recombinant strains are then used for quantitative trait locus (QTL) analysis to define chromosomal regions contributing to the phenotype of interest\(^{(28)}\).

Such a genomic region may harbour a large number of genes and one or several of these may be associated with the selected phenotype. The display of the phenotype could result from the expression of a polymorphism of a target gene(s) and an altered functionality of the gene product. The altered expression pattern of other genes may be a downstream consequence of this changed function.

**Genetic predisposition to alcohol preference and to the effects of alcohol**

One approach to identify specific genes and pathways associated with a tolerance of the acute effects of alcohol or preference for alcohol consumption is to compare the baseline gene expression of rodent strains with different phenotypes (Table 1). Bhave et al. investigated the expression levels of gene variants encoding enzymes concerned with alcohol metabolism\(^{(29)}\). The expression of alcohol dehydrogenase gene variants was measured in whole-brain extracts of two alcohol-naive mouse strains with different alcohol preference and was shown to be associated with the selected behaviour\(^{(29)}\).

Tabakoff et al. used mice strains bred for differences in tolerance to acute effects of alcohol and compared whole-brain gene expression between representative individual animals\(^{(30)}\). The data were processed using several methods of data filtering followed by statistical analyses. A group of genes, which were concerned with glutamate receptor activity, was revealed. These mapped to previously identified QTL. The candidate genes included those encoding the N-methyl-D-aspartic acid (NMDA) receptor and the glutamate receptor 62 protein. Carr et al. aimed to identify genes located within one specific, alcohol-related QTL\(^{(31)}\).

The researchers investigated gene expression in the NAC, PFC, amygdala (AMYG), hippocampus and striatum from congenital rat strains differing only in one alcohol preference-related QTL region on chromosome 4 and exhibiting the selected phenotype\(^{(31)}\). Glutamate receptor expression was altered in multiple brain regions, while combined analysis, across all regions, implicated protein phosphatase C signalling and neuropeptide Y (NPY) in alcohol preference\(^{(31)}\). The involvement of glutamatergic neurotransmission, particularly receptors, in the acute effects of alcohol is well established\(^{(20)}\). Protein phosphatase C and NPY signalling contribute to several aspects of alcohol-related behaviour including sensitivity to acute effects of alcohol and control of alcohol consumption\(^{(20,32)}\).

In a larger study, the baseline whole-brain gene expression of three selected lines, and six isogenic strains of mice, which again differed markedly in voluntary alcohol consumption, was compared\(^{(33)}\). Genes associated with the mitogen-activated protein kinase (MAPK) signalling pathways which regulate activity of cytoskeletal elements and mediate the influence of NFκB on transcription had predominantly higher expression in alcohol-preferred animals and were proposed to be linked to the alcohol-related behaviour. Additionally, a group of candidate genes mapped to an alcohol preference QTL on chromosome 9.
Inbred Long-Sleep and Short-Sleep mice differentially express a number of genes thought to be implicated in sensitivity to the effects of ethanol\(^{34}\). A promoter analysis of genes differentially expressed in the cerebellum in these strains identified cAMP response element binding (CREB) signalling to be a common feature of many of these genes\(^{35}\). In a study comparing gene expression levels in the PFC of alcohol-preferring and non-preferring rats, MAPK and CREB signalling was again associated with alcohol-related behaviour\(^{36}\). Additionally genes concerned with mitochondrial function were differentially expressed between the rat strains\(^{36}\). NFκB, Jun and CREB signalling as well as mitochondrial function are involved with cell survival and apoptotic events. The signalling pathways and transcription regulatory elements identified by these studies might modulate the detrimental effects of alcohol on the brain.

Arlinde et al\(^{37}\) compared gene expression in the NAC, hippocampus, cingulated cortex and AMYG of non-congenital alcohol-preferring, alcohol non-preferring and non-selected Wistar rat strains using Affimrix arrays containing probes for 1000 genes. Forty-eight genes were found to be differentially expressed between the alcohol-preferring and alcohol non-preferring rat strains. Notably, genes concerned with MAPK signalling were induced in the NAC of alcohol-preferring rats and the levels of a group of transcripts involved with the regulation of cell structure were altered in the AMYG, pointing to region-specific pathways involved in alcohol preference. With the exception of NPY, which was differentially expressed in the hippocampus, none of these genes is located near known QTL for alcohol preference or consumption in rats. This discordance might have been due to the relatively small number of genes analysed. Alternatively, regulatory elements of the genes identified might be located in such regions of the MDS. The NAC and AMYG are involved in drug-related learning and craving. The differential expression of genes associated with plasticity within these regions of the MDS may therefore reflect a heightened predisposition for the development of dependence and modulate craving in alcohol-preferring animals.

**Effect of alcohol administration on gene expression in the dopaminergic mesocorticlimbic system**

To complement the comparison of baseline gene expression in rodent strains with distinct alcohol-related behaviours, the differences in the effect of alcohol administration on gene expression in several regions of the MDS in these strains have been investigated (Table 2). Kerns et al. exploited the differences in alcohol-related behaviour of C57BL/6 (B6) and DBA/2J (D2) mice: B6 mice drink more alcohol than D2 mice and display a larger locomotor response to acute alcohol exposure\(^{39}\). Gene expression in
Table 2. Effect of alcohol administration in the animal model

<table>
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<th>Molecular pathways (candidate genes)</th>
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<td>Transcription factor (Sp1 and NFκB) signalling</td>
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<td>Intermittent, chronic alcohol administration</td>
<td>NAC, medial PFC, AMYG</td>
<td>Repunte-Canonigo et al. (2007) (46)</td>
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<tr>
<td>Protein kinase A signalling and neurotransmission (GABAergic and glutamatergic transmission) in all three brain regions; dopaminergic transmission in medial PFC and NAC</td>
<td>Rat</td>
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<td>Chronic alcohol self-administration compared with natural reward (saccharine)</td>
<td>NAC</td>
<td>Rodd et al. (2008) (48)</td>
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</table>

VTA, ventral tegmental area; NAC, nucleus accumbens; PFC, prefrontal cortex; PI, phosphatidylinositol; AMYG, amygdala; EAAT1, excitatory amino acid transporter 1; GluR2, glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit 2; MAPK, mitogen-activated protein kinase; GABA, γ-aminobutyric acid.
the VTA, NAC and PFC before and after acute intraperitoneal alcohol injection was compared. Differentially expressed genes were then mapped to previously established alcohol-related QTL in a comprehensive approach to identify genes and signalling pathways associated with the alcohol response. Expression of genes associated with differentiation was altered in the VTA in both strains. Alcohol also affected transcript levels of several growth factors and developmental genes in the NAC, although the response varied in the different strains. In D2 mice, for example, brain-derived neurotrophic factor and genes associated with neurotransmission were altered in the NAC and genes associated with glucocorticoid signalling, neurogenesis and myelination were influenced in the PFC. Hence, a striking theme common to the brain regions investigated in this study was the altered expression of genes associated with plasticity, which suggests neuroplastic alterations within the MDS in the response to acute alcohol administration. Differentially expressed gene sets associated with neuroplasticity and myelination also mapped to chromosomal regions harbouring several ethanol behavioural QTL(39). The studies highlight the importance of neuroplasticity-related pathways within several regions of the MDS which may modulate the susceptibility to excess alcohol consumption.

As emphasised by Singh et al. the cause and effect between differential gene expression and changed phenotypes remain to be established(40). Testing the co-segregation of the phenotype of interest and expected gene expression level in independent genetic crosses or using recombinant inbred mice strains could strengthen this link(40). Towards this aim, Hu et al. utilised thirty BXD recombinant inbred strains of mice, twenty inbred strains of mice, and two replicate lines of mice selectively bred for differences in functional tolerance to acute alcohol to compare baseline gene expression(41). Differentially expressed genes were mapped to known QTL. The level of selected gene expression was correlated to the phenotype and the heritability was monitored. Eight genes were identified as candidate genes for acute alcohol tolerance. They included K voltage-gated channel, Shab-related subfamily, member 1, which dampens excitability of neurons and serine/threonine kinase, which can influence glutamatergic transmission. In combination with previous studies(30,31,38,39), the results again strongly imply neuron excitability and particularly glutamatergic transmission as a target for acute alcohol. The identification of similar genes and signalling pathways associated with alcohol-related behaviours in various rodent models highlights the benefits of combining gene expression studies with genetics to reveal gene networks in the brain that may be involved in a predisposition to alcohol abuse and addiction.

Inbred rodent strains of identical genetic background were utilised to establish the impact of alcohol administration on brain gene expression. The aim of these studies was to mimic neuroadaptations associated with alcohol tolerance and dependence. Rulten et al. investigated the impact of acute alcohol on midbrain gene expression in mice, 2 h following a single intraperitoneal ethanol dose(42). Genes concerned with transcription factor Sp1 and NFκB signalling pathways were found differentially expressed. Sp1 and NFκB expression were also altered in mice fed an escalating ethanol diet for 3 weeks(42), implicating these pathways in acute as well as chronic alcohol responses.

Wistar rats offered a 12 % alcohol solution as the only source of liquid for 12 weeks were used to elucidate the effect of chronic alcohol exposure on gene expression in the hippocampus(43). The expression of sets of genes encoding oxidoreductases and proteins concerned with membrane trafficking was altered. This may reflect oxidative stress and an interference with membrane function. Rimondini et al. investigated the impact of 7 weeks of repeated cycles of alcohol vapour treatment and withdrawal on gene expression in the AMYG and the cingulate frontal cortex of Wistar rats(44). This treatment paradigm induces increased voluntary alcohol consumption. In the AMYG, transcript levels of insulin-like growth factor 2 and genes involved in phosphatidylinositol 3-kinase signalling were affected. The role of these genes in responses to alcohol dependence within the AMYG is unknown. Expression of genes associated with MAPK signalling was altered in the PFC. Once more, induction of NFκB confirms adverse effects of alcohol on this brain region. Elevated levels of genes concerned with neurotransmission were revealed in the cortex and included the cannabinoid receptor CB1. Accordingly, administration of cannabinoid receptor antagonist SR141716A reduces alcohol consumption in strains of alcohol-prefering rats, presenting a pharmaceutical target for the treatment of alcoholism(35). The induction of the excitatory glutamate transporter 1 (known as EAAT1 or GLAST) and glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit 2 observed in this study(43) may represent an adaptation to altered glutamatergic transmission. Repunte-Canongia et al. utilised the same treatment and animal model to investigate the impact of intermittent alcohol administration on the transcriptome of the NAC, medial PFC and AMYG(46). An observed alteration in expression of genes regulating protein kinase A signalling in all three brain regions could have a downstream modulatory effect on CREB signalling. A number of dopamine-regulated transcripts were differentially expressed in the medial PFC and NAC, which might indicate an alteration in dopaminergic drive. Together with a region-specific change in expression of GABA and AMPA receptor subunits, these results suggest extensive but specific adaptive alteration to neurotransmission in response to chronic alcohol administration.

**Effect of alcohol self-administration on gene expression in the dopaminergic mesocorticolimbic system**

Such involuntary treatment regimens might induce severe stress responses and as such not fully reflect voluntary and intermittent excessive drinking in human alcoholics. Saito et al. minimised these variables by utilising two mice strains with different alcohol preference. The researchers applied a 9-d voluntary self-administration model with an escalating regimen of alcohol concentration(47). This treatment regimen failed to produce significant changes to gene expression and may have been too mild to produce dependence. Rodd et al. investigated the impact of operant ethanol self-administration on gene expression in
NAC inbred alcohol-preferring rats by comparing differences and commonalities between the effect of a natural reward and alcohol (56) (Table 2). The expression of genes encoding proteins associated with the synapse and homeostasis was altered in response to ethanol or saccharin. Genes associated with neurogenesis were differentially expressed between ethanol and the natural reward administration. These genes may therefore be sensitive to the specific incentive.

Sex-specific action of alcohol

Male rodents are mainly utilised for gene-expression studies investigating the effect of alcohol exposure. However, the effect of sex was revealed in a recent study on gene expression in male and female rodent brains after 72 h exposure to ethanol vapours followed by 8 h withdrawal (57). The study highlights significant sex-specific differences. In the female the treatment regimen had an impact on expression levels of genes involved in cell death, while altered expression of genes concerned with protein modification and degradation featured in male brains. Histopathological analysis of brain damage confirmed that the observed cell death was sex specific.

Nicotine-responsive gene expression in the animal model

Effects of nicotine administration on gene expression in the dopaminergic mesocorticolimbic system

To a lesser extent than alcohol, the effect of acute and chronic nicotine on gene expression in the brain has also been investigated in rodents (Table 3). In mice, acute nicotine altered the expression of genes associated with plasticity in the VTA (58). Ras homologue gene family, member A, a gene involved in structural remodelling, was then selected from this gene set for a case–control gene association study in human subjects and found to be significantly associated with smoking initiation (59). Intermittent nicotine administration followed by withdrawal elicited significant locomotor sensitisation in quasi-congeneric RQI and donor BALB/cJ mice but not in C57BL/6ByJ mice. Gene expression was monitored in the VTA of these strains (51). Nicotine-responsive genes were concerned with signal transduction, intracellular protein transport, proteasomal ubiquitin-dependent protein catabolism, and neuropeptide signalling. While some nicotine-responsive genes and pathways overlapped, there were distinct differences between the strains. This suggests common regulatory mechanisms across inbred strains but also that a small effect of sex was revealed in a recent study on gene expression in male and female rodent brains after 72 h exposure to ethanol vapours followed by 8 h withdrawal (57). The study highlights significant sex-specific differences. In the female the treatment regimen had an impact on expression levels of genes involved in cell death, while altered expression of genes concerned with protein modification and degradation featured in male brains. Histopathological analysis of brain damage confirmed that the observed cell death was sex specific.

An earlier microarray study established that repeated nicotine treatment affects several signal transduction pathways in the PFC, NAC, VTA and AMYG (52) of Holtzman rats. In the PFC, VTA and AMYG, genes involved in MAPK signalling were revealed as nicotine sensitive. mRNA levels of genes involved in phosphatidylinositol signal transduction were affected in the NAC, VTA and AMYG in a region-specific manner. Transcript levels of genes associated with the epidermal growth

### Table 3: Effect of nicotine in the animal model

<table>
<thead>
<tr>
<th>Molecular pathways (candidate genes)</th>
<th>Strain characteristics</th>
<th>Experimental design</th>
<th>Brain region</th>
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<tbody>
<tr>
<td>Plasticity (RhoA)</td>
<td>One inbred strain</td>
<td>Repeated intermittent nicotine administration</td>
<td>VTA, PFC, AMYG, NAC</td>
</tr>
<tr>
<td>MAPK signalling in PFC, VTA and AMYG, PI signalling in the AMYG</td>
<td>Two strains, which differ in a number of nicotine-related behaviours</td>
<td>One inbred strain</td>
<td>PFC</td>
</tr>
<tr>
<td>Proteasome–ubiquitin system</td>
<td>One inbred strain</td>
<td>Chronic systemic nicotine administration</td>
<td>PFC and medial basal hypothalamus</td>
</tr>
<tr>
<td>Amyloid precursor protein in AMYG and hippocampus; Amyloid precursor-like protein in AMYG</td>
<td>One inbred strain</td>
<td>Chronic oral nicotine administration on genes</td>
<td>AMYG, hippocampus</td>
</tr>
<tr>
<td>EGFR, epidermal growth factor receptor</td>
<td>One inbred strain</td>
<td>MAPK-, small GTP-mediated signal transduction in VTA; cell cycle in NAC and PFC; ubiquitination in parietal cortex</td>
<td>Mouse</td>
</tr>
<tr>
<td>RhoA, Ras homologue gene family, member A, VTA, ventral tegmental area, MAPPK, mitogen-activated protein kinase, PFC, prefrontal cortex, AMYG, amygdala, PI, phosphatidylinositol; NAC, nucleus accumbens;</td>
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https://doi.org/10.1017/S0954422409990114
factor receptor (EGFR) signalling pathway were altered in the AMYG. In a follow-up study using pathway-focused arrays these signalling cascades were investigated further in the PFC, striatum, medial basal hypothalamus, AMYG and VTA of the rat brain in response to subacute and chronic systemic nicotine administration\cite{53}. Rats were killed at 3, 7 and 14 d of nicotine administration. The observed changes in gene expression were highly region-specific and dynamic over the time studied. Thus, genes involved in signalling events were up-regulated at day 3 in the AMYG and at day 7 in the NAC and down-regulated over time in the VTA. These changes may be an adaptive process to chronic nicotine treatment. The expression of genes associated with the cytoskeleton was reduced after 14 d in the AMYG. Such genes were altered in the opposite direction in the PFC, which may indicate region-specific restructuring events in response to chronic nicotine. Genes concerned with inhibitory GABAergic neurotransmission were down-regulated while those concerned with glutamatergic transmission were induced, indicating an increased excitatory drive in response to systemic nicotine administration. In the medial basal hypothalamus a reduction of transcripts associated with ubiquitination was evident over time. These alterations were further explored in the PFC and medial basal hypothalamus in a third study by the same research group, again utilising customised pathway-focused microarrays\cite{54}. The 14 d nicotine administration applied in this study modulated expression of genes associated with the proteasome–ubiquitin system in a region-specific manner. The authors hypothesise that the observed changes may be caused by oxidative stress or changes to synaptic activity. Together with previous findings\cite{51} this strongly suggests that nicotine has distinct effects on cellular protein homeostasis within the MDS.

Another research group investigated gene expression in response to repeated intermittent nicotine administration on the parietal cortex of mice and identified altered expression of a set of transcription factors\cite{55}. Subsequently they utilised in situ hybridisation to confirm elevated levels of the immediate early genes, Egr-1 and Egr-2, as well as the orphan nuclear receptor, Nr4a1, in the parietal cortex and the hippocampus. The transcription factor activities of the protein products of these nicotine-sensitive genes could induce expression of a large number of other genes in a secondary response.

**Effect of genetic predisposition on expression of nicotine-sensitive genes in the dopaminergic mesocorticolimbic system**

Recently, a comprehensive study investigated genetic predisposition and the effect of chronic nicotine gene expression in five regions within the MDS in two mice strains\cite{56}. C3H/HeJ and C57BL/6J mice differ in a number of nicotine-related behaviours. C3H/HeJ mice self-administer less nicotine\cite{57} and a low dose of nicotine increases locomotor activity in C3H/HeJ mice but depresses it in C57BL/6J mice\cite{58}. C3H/HeJ mice develop tolerance only at higher doses of chronically infused nicotine\cite{59}. A pathway-focused microarray and gene ontology analysis were used to compare the impact of chronic nicotine administration on gene expression in the NAC, PFC, VTA, AMYG and hippocampus. In both strains nicotine had an impact on genes concerned with neurotransmission in the NAC, PFC and VTA. The expression of some of these genes was affected in inverse directions and included the nicotinic acetylcholine receptor α4 subunit. Genes concerned with glutamatergic transmission were altered in the NAC and PFC in a strain-specific manner. These changes were consistent with differences in sensitivity to nicotine and nicotine-related behaviours in the two strains. In C3H/HeJ mice, nicotine induced expression of genes concerned with integrin-, MAPK- and small guanosine triphosphatase-mediated signal transduction in the VTA, which points to structural alterations in response to chronic nicotine within this core region of the MDS. Genes associated with the cell cycle were induced in the NAC and down-regulated in the PFC in response to chronic nicotine in C3H/HeJ mice. Products of these genes may have a role in synaptic plasticity. Genes concerned with protein ubiquitination and catabolism were again affected in the hippocampus in C57BL/6J mice.

**Protective effects of nicotine**

Epidemiological and molecular studies have indicated that nicotine may have neuroprotective potential against Alzheimer’s and Parkinson’s diseases\cite{60,61}. To investigate this further, the effect of chronic oral nicotine administration on genes encoding amyloid β and related proteins was studied in brain regions of mice using a pathway-specific microarray\cite{62}. The evident up-regulation of genes for amyloid precursor protein in the AMYG and hippocampus and amyloid precursor-like protein 2 in the AMYG may contribute to the protective effects of nicotine against Alzheimer’s disease.

**Common molecular targets of alcohol and nicotine within the dopaminergic mesocorticolimbic system**

Nicotine interacts with specific nicotinic acetylcholine receptors in the first instance. However, these studies highlight that a number of molecular pathways are implicated in the events following acute and chronic nicotine exposure. Importantly, when comparing the effects of alcohol and nicotine in the animal model, an overlap in affected signalling pathways, particularly the MAPK and phosphatidylinositol signalling cascades, is evident. Additionally, one may ponder if rodents and the applied treatment regimens fully model human alcohol and/or nicotine abuse and addiction. An important caveat in the animal model is the difficulty in reflecting changes occurring in the human MDS over a lifetime of abuse and relapse.

**Gene expression profiling of the human prefrontal cortex of chronic alcoholics**

Changes to gene expression and molecular pathways within the MDS associated with long-term alcoholism have been
Gene expression in the human brain

Comparative gene expression profiling of regions of the dopaminergic mesocorticolimbic system in chronic alcoholics

In a series of microarray studies our research group profiled the PFC (BA 9), NAC and VTA of long-term alcohol abusers using a consistent method of RNA extraction, linear amplification, microarray platform, hybridization, as well as data analysis. In the studies of the BA 9 region of the PFC and the NAC a two-colour design was used, while for expression screening of the VTA a universal reference was applied. The results of each microarray study were verified by real-time PCR on selected genes.

Comparing differentially expressed genes between the three studies, the highly region-specific action of alcohol was a striking observation. No alcohol-sensitive gene was common between the three studies and only 4% between any two. Functional clusters of alcohol-sensitive genes identified in the NAC and VTA were clearly distinct from the PFC. With the exception of a group of genes concerned with transcription in the VTA, none of the main themes present in the PFC was observed in the VTA or NAC. The most pronounced functional group in the VTA contained genes associated with cell signalling. In the VTA and the NAC a number of genes were associated with synaptic and/or structural plasticity.

In early microarray studies, genes associated with alcohol abuse were grouped manually into functional groups. More recently, functional data mining of large microarray datasets using independent softwares has been applied as an unbiased tool to reveal the biology underlying a dataset. The web-based software Database for Annotation Visualisation and Integrated

identified by investigating human post-mortem brain tissue of chronic alcoholics and control cases. The PFC (particularly Brodmann area 9; BA 9) is involved in working memory and is connected to the hippocampus, association areas and parietal cortex, thus providing information on the context of stimuli for higher-order sensory processing. Alcohol abuse has an adverse impact on this brain region. Older alcoholics exhibit a decrease in white matter volume and a significant neuronal loss in grey matter. The PFC was the first brain region in human subjects to be investigated by gene expression profiling by two research groups.

In early microarray studies on the PFC of the chronic alcoholic, restrictions in human brain tissue resources required pooling of mRNA samples. These studies revealed changes to the expression of several hundred genes in the chronic alcoholic, including genes concerned with myelination. Advances in technology later enabled investigators to profile the transcriptome of the PFC in individual samples. These studies also identified an alteration in the expression of genes concerned with myelination, which may point to an interference of alcohol with membrane integrity and neurotransmission. Further, in two studies the expression of transcription factors, including the immediate early genes, activator protein 1 (AP-1) and CREB, was sensitive to chronic alcohol abuse. These results are in accordance with expression profiling in the animal models and may indicate an alteration to cell fate in the PFC of the long-term alcoholic. Certainly, loss of neurons is a characteristic of the PFC of the alcoholic.

The detrimental effects of alcohol on the brain may be an effect of the direct toxicity of alcohol and its first metabolite acetaldehyde. In addition, changes in the balance of inhibitory and excitatory neurotransmission in the cortex may elevate neuronal sensitivity to glutamate during withdrawal to levels causing excitotoxicity. Interestingly and similar to the animal model, one study revealed a robust induction of the glial glutamate transporter, excitatory amino acid transporter 1, in the PFC of the human alcoholic. Consequently, the altered expression of this transporter was confirmed at the protein level. Taken together these studies point to a protective role of excitatory amino acid transporter 1 against the potentially cytotoxic glutamate level following withdrawal. Chronic ethanol treatment of rats results in DNA damage and an increased level of heat shock proteins in the cortex. Correspondingly, in the PFC of the human alcoholic, genes associated with DNA repair and those encoding a number of heat shock proteins and free radical scavengers were induced. The down-regulation of mitochondrial genes including those associated with electron transport or energy production was revealed in two studies. Mitochondrial function was again implicated in the animal model of alcohol preference. These changes indicate that disruption of mitochondrial function may be a possible source of oxidative stress. In summary, microarray gene expression studies on the PFC of human alcoholics identified gene groups associated with a disturbance of membrane function, transcription and oxidative stress and cell death and survival. The alteration in transcription observed in these genes may reflect the neuronal cell loss evident in the long-term alcoholic.

Comparisons of the various studies on the human PFC reveal few common specific alcohol-sensitive genes and only partial overlap in functional gene groups. The inconsistency between the studies emphasises the need for strictly controlled experimental set ups. As reviewed previously, uncharacterised cases as well as experimental factors may play a role in the divergent results. Possible confounding factors in human studies include mode of death, tissue pH, white matter contamination, post-mortem interval, onset of drinking, age, intoxication state at death, co-morbidity with other drugs of abuse or with underlying psychiatric disorders. Distinct differences in experimental design, application of various microarray platforms together with platform-specific methods to treat and hybridise input RNA sample and the choice of method of analysis may lead to pronounced differences in the results. Despite these caveats, the studies have revealed alcohol-sensitive gene groups highly relevant to the pathology evident in the PFC of the chronic alcoholic. The accordance of genes and gene networks with those identified in expression profiling using rodent models highlights that the two approaches are complementary in the identification of alcohol-responsive molecular pathways.
Analysis (DAVID/EASE; National Cancer Institute at Frederick, Frederick, MA, USA; http://david.abcc.ncifcrf.gov) groups genes into specific categories considering their multi-functionality(81–83). Further, the software identifies over-represented annotation terms and biological themes using χ statistics and maps genes to canonical Kyoto Encyclopedia of Genes and Genomes pathways(82). DAVID/EASE analysis (National Cancer Institute at Frederick) on the datasets of differentially expressed genes was performed for the VTA, PFC (Table 4) and the NAC (Table 5) to reveal over-represented annotations and functional gene groups within the three brain regions. Groups of genes associated with transcription or apoptosis were enriched in the PFC only. Over-representation of genes coding for proteins involved with modification of the extracellular cellular matrix was unique to the NAC. For example, tissue inhibitor of metalloproteinase (TIMP) 1 and TIMP4 were induced, while TIMP2 was down-regulated. This suggests an alteration to extracellular matrix homeostasis. Additionally, genes concerned with cell adhesion featured in a separate cluster. They included integrin, α 6, T-cell lymphoma invasion and metastasis 1, contactin-associated protein-like 2 and ninjurin 2. Genes associated with tissue development, neuronal cell morphology and neuron morphogenesis and differentiation were also tied into multiple independent clusters. These functional themes strongly suggest structural plasticity in the NAC of the chronic alcoholic. To a lesser extent, genes which are expressed during structural plasticity were also identified as enriched in one functional cluster in the VTA(63). Of interest within these functional clusters were molecular targets common to both the NAC and VTA. In both regions, chronic alcohol abuse changed the expression of genes participating in integrin-mediated alteration of the cytoskeleton. Enduring, structural plasticity within the NAC and VTA in response to repeated drug administration has been observed in animal models(84). These changes are thought to underlie the persistence of craving and relapse. Interestingly, comparing studies on human and animal models, a meta-analysis identified genes concerned with structural plasticity to feature in the group of top candidate genes for alcoholism(85). Further, in the human VTA, genes concerned with neurotransmitter transport and release were significantly over-represented amid the alcohol-sensitive genes identified(63). In conclusion, comparative expression profiling of distinct regions of the human MDS revealed a region-specific impact of alcohol. Structural plasticity in the NAC and VTA may underlie enduring alterations to connectivity within these two brain regions and open up novel avenues for drug research.

Table 4. Functional enrichment clustering of genes associated with alcohol abuse in the human prefrontal cortex (PFC)*

<table>
<thead>
<tr>
<th>Functional group</th>
<th>n</th>
<th>Category</th>
<th>Annotation term</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell fate</td>
<td>26</td>
<td>GOTERM_BP_ALL</td>
<td>Induction of apoptosis</td>
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<td>3.1 × 10⁻²</td>
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<tr>
<td>Transcription</td>
<td>50</td>
<td>INTERPRO</td>
<td>Zn finger, C2H2-type/integrase, DNA-binding</td>
<td>6</td>
<td>7.4 × 10⁻³</td>
</tr>
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<td>Gene product location</td>
<td>33</td>
<td>GOTERM(CC_ALL)</td>
<td>Integral to plasma membrane</td>
<td>12</td>
<td>3.0 × 10⁻²</td>
</tr>
</tbody>
</table>

* Differentially expressed genes were identified by comparing gene expression in the PFC of chronic alcoholics and control cases. This gene list was subjected to functional enrichment clustering using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov). Clusters with at least one significant annotation term are presented. Annotation terms are listed with associated Gene Ontology (www.geneontology.org), InterPro (www.ebi.ac.uk/interpro/) and SwissProt/PIR/Uniprot (www.ebi.ac.uk/swissprot/, http://www.uniprot.org) categories.

Tobacco co-abuse in chronic alcoholics

There is a strikingly high coincidence between alcoholism and smoking. Alcoholism is more prevalent in smokers than non-smokers and 80–90% of alcoholics are heavy smokers compared with 33% of the global population(11). Smoking cessation is more difficult in former and current alcoholics and cessation of smoking improves the chances of a reduction in alcohol consumption(12). In addition, smoking may act as a precipitant of relapse to alcoholism(12). In co-morbid cases, drinking elevated craving for cigarettes and smoking cues increased the desire for alcohol(86). Recent meta-analysis of linkage and association studies have identified candidate genes which may predispose an individual to addiction to more than one drug(57). These included genes involved in dopamine and serotonin neurotransmission and in drug metabolism. Potential predisposition to alcohol and nicotine dependence predominated(87). Despite the potential for extensive interactions, most studies on gene expression within the MDS of the human alcoholics using high-throughput genomics have not taken account of the common co-morbidity of smoking and alcoholism(64–69). Compelling evidence arising from two recent studies identified that smoking has a profound impact on alcohol-related gene expression in the human. In the PFC and VTA, the expression levels of selected alcohol-sensitive genes were established in an extended case set of alcoholics with or without smoking co-morbidity, non-alcoholic smokers and non-smoking, non-drinking controls(63,76). In the PFC, smoking alone altered expression of apolipoprotein D (APOD) and midkine and increased variance in the expression of TIMP3 and excitatory amino acid transporter 1(76). The neurotrophic factor, midkine, is induced after ischaemia(88). TIMP3 facilitates doxorubicin-induced apoptosis in neuronal culture(89). APOD function is yet unknown, but the expression of this gene was altered in schizophrenics and patients with bipolar disorder(90). Smoking is associated with impairment of cognitive functions(91) and exacerbates alcohol-induced neuronal injury(92). Taken together, these studies emphasise that smoking alone may have a negative effect on cell viability in the PFC.

In the VTA, smoking and alcohol interacted to alter the expression of excitatory amino acid transporter 2 and smoking alone induced expression of vesicular glutamate transporters 1 and 2(63). These changes indicate a potentiation of glutamatergic neurotransmission in response to smoking(93). Glutamatergic transmission is vital for the control of the VTA and may also be critical to the weighting of novelty and importance of a stimulus, an essential output...
of this brain region. Therefore enduring plasticity within the VTA may be a major molecular mechanism for the maintenance of smoking addiction and alcohol, nicotine and co-abuse have distinct impacts on glutamatergic transmission with important implications for the control of this core mesolimbic structure. Interestingly, preliminary clinical studies in subjects dependent on nicotine\(^\text{94}\) and alcohol\(^\text{95}\) show that modulation of glutamatergic function may be a useful tool for initiation of abstinence and prevention of relapse.

These studies of core regions of the human MDS emphasise the necessity to account for smoking as a confounding variable in studies of alcoholism both with regards to dependence but also as a component of alcohol-related brain damage. Future large-scale gene expression studies carefully delineating the effect of alcohol from that of smoking in the human MDS may further enhance our understanding of the impact of alcohol and smoking on the human brain.

### Protein expression studies

Transcription profiling provides a snapshot of the mRNA spectrum of tissue under study and indicates the types of changes that may be taking place. However, mRNA is not the functional endpoint of gene expression and often mRNA changes that may be taking place. However, mRNA is not

<table>
<thead>
<tr>
<th>Functional group</th>
<th>n</th>
<th>Category</th>
<th>Annotation term</th>
<th>n</th>
<th>P</th>
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<td></td>
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<td>Baseline membrane</td>
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<td>1.2 \times 10^{-2}</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>GOTEMP BP ALL</td>
<td>Localisation</td>
<td>25</td>
<td>1.3 \times 10^{-2}</td>
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* Differentially expressed genes were identified by comparing gene expression in the NAC of chronic alcoholics and control cases. This gene list was subjected to functional enrichment clustering using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/). Clusters with at least one significant annotation term are presented. Annotation terms are listed with associated Gene Ontology (www.geneontology.org), InterPro (www.ebi.ac.uk/interpro/) and SwissProt/PIR/Uniprot (www.ebi.ac.uk/swissprot/, http://www.uniprot.org) categories.

Proteome of MDS regions\(^\text{98}\). Two-dimensional gel electrophoresis, which separates proteins, firstly on isoelectric charge and then by molecular mass, can resolve over 1000 protein species. This methodology is then coupled with matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) to confirm identity of a limited number of protein species. Currently, these analyses have been restricted to soluble proteins of mid-range molecular weight expressed at a relatively high level.

In an initial study using pooled samples from four individuals, Lewohl et al. reported changes in the levels of 183 proteins in the PFC\(^\text{98}\). More recently in a detailed comparative study, Matsumoto and colleagues documented changes in the proteome of the BA 9 region of the alcoholic brain\(^\text{99}\). Further, the proteome of the grey matter was compared with that of the underlying white matter\(^\text{100}\). In these studies, the influence of confounding liver disease was also monitored.

The level of several thiamine-dependent enzymes was changed in both grey and white matter. Further, almost 50 % of the alcohol-sensitive proteins were metabolic enzymes, many of which are involved in energy transduction such as those involved in glycolysis and the tricarboxylic acid cycle. Disruption of these pathways, possibly emanating from a deficiency in thiamine metabolism, has several implications. Deprivation of ATP supply may lead directly to a loss of cellular viability but also disruption of the thiamine-dependent enzymes of the pentose phosphate pathway may reduce the supply of reducing equivalents and therefore the cells’ ability to combat oxidative stress. Many of the changes in protein expression in the alcoholic were enhanced in cases with liver disease\(^\text{101}\).
Gene expression studies on the human dopaminergic mesocorticolimbic system of heroin and cocaine addicts

The impact of other drugs of abuse, in particular heroin and cocaine, on gene expression in the human NAC has been studied by Albertson et al., using the Affymetrix platform\(^{(102,103)}\). Seven heroin abusers were compared with matched controls and approximately 1000 genes were identified as differentially expressed\(^{(102)}\). Of particular interest was the down-regulation of genes encoding pre-synaptic proteins. These proteins are involved in aspects of neurotransmitter release, including vesicle storage, release and recycling. The results may indicate decrements in neurotransmission within the NAC of the chronic heroin abuser. In a second study, Albertson et al. compared gene expression of ten chronic cocaine abusers with matched controls\(^{(103)}\). Only forty-nine genes were identified to be differentially expressed in the cocaine abusers. Therefore and in contrast to the heroin study and to our studies on the NAC of chronic alcoholics, cocaine exposure might affect only a select group of genes in the human NAC. In both studies, differentially expressed genes were manually assigned to diverse functional groups. In the cocaine abusers, a number of genes were glia-specific and included genes involved in myelination. Particularly, a decrease in expression of myelin basic protein, myelin-associated oligodendrocyte basic protein and proteolipid protein was revealed. Myelin basic protein and proteolipid protein constitute about 80% of myelin in the central nervous system and a dysregulation of myelin in the cocaine abuser is consistent with white matter pathology in these addicts\(^{(104–106)}\). These studies highlight the specific and diverse actions of different drugs of abuse on the human NAC.

The proteome of the NAC has also been explored in cocaine abusers\(^{(107)}\) and in self-administering Rhesus monkeys\(^{(108)}\). Consistent with the concept of drug-induced plasticity in this region of the human brain, several proteins associated with synaptic plasticity were found to have altered levels in cocaine abusers. In addition, altered metabolic flux was suggested by changes in the level of proteins involved in energy metabolism whilst other changes were suggested to reflect increased oxidative stress. In the subsequent study of the primate model, protein changes indicated a dysregulation of proteins related to cell structure, signalling and metabolism including energy generation in the mitochondria.

Future studies

Rodent models of drug-seeking behaviours and drug dependence continue to be developed. Studies of these models coupled with advances in rodent genetics will provide important information on the mechanism of action at the cellular and molecular level. It will also be important to continue with exploration of the human brain in parallel with these animal studies. Currently, a major difficulty is matching the physiological conditions of each human case. In addition to the variation in lifestyle pre-mortem, differences in post-mortem treatment of tissues are a confounding factor. However, continued improvement in public awareness, documentation and tissue treatment in the various Brain Banks worldwide should result in increased numbers of well-controlled cases for future studies.

Expression profiling experiments, by nature, generate a large amount of data. Application of analysis softwares such as DAVID (National Cancer Institute at Frederick; http://david.abcc.ncifcrf.gov), Pathway Assist by Stratagene (Agilent Technologies, Santa Clara, CA, USA) and others can indicate underlying biological themes. Recent advances in tools to group genes by variation of expression across individual samples have made possible the exploration of gene networks\(^{(109)}\). This type of analysis has been directed at diverse biological problems, for example, evolution of the human cortex\(^{(110)}\) and genes differentially expressed during the progression of Alzheimer’s disease\(^{(111)}\). Similar approaches to group differentially expressed genes in the drug-affected MDS may reveal expression-related gene groups and genes central in these networks (hub genes) which may have key roles. This will give an insight into the organisation of drug-sensitive genes and possible new pharmacological targets.

Recent proteomic analyses have significantly contributed to understanding the pathophysiology of the complicated disease which is alcoholism. The data from these studies illustrate both the power and limitations of current technologies for proteomic analyses. Clearly these approaches identified important biologically relevant changes at the end point of gene expression; however, available technologies have limited ability to detect high- or low-molecular-weight proteins, and hydrophobic or insoluble proteins associated with membranes. These proteins are often important in understanding the mechanisms of central nervous system diseases. Advances in protein separation and identification have the potential to analyse expression levels of proteins, including neurotransmitter receptors and transporters that have not been examined using current techniques.

Changes in the transcriptome of the alcoholic brain may be a result of many influences. Some changes in expression may be a result of drug toxicity, some may reflect neuroadaptations associated with the development of tolerance and others may result from genetic variation. The latter may have importance in predisposition to drug dependence. Animal studies relating changes in gene expression to behaviours and to QTL maps have been invaluable in identifying potential inheritable genetic factors. Recent genome-wide association studies of alcohol\(^{(112–115)}\) and nicotine dependence\(^{(116,117)}\) have identified several potential markers. Further meta-analyses comparing human gene expression and gene association studies will greatly aid the identification of genes and gene networks crucially involved in the predisposition to alcohol, tobacco and co-abuse in humans\(^{(87)}\). One recent study resulted in a systems biology network model derived by ontology-based computational genotype–phenotype association analysis of the pharmacogenetics of nicotine addiction and treatment\(^{(118)}\). The model encoded a disease risk model capturing the underlying biological systems and interactions with the environment. Such combined studies and analyses may reveal novel candidates to improve current treatment regimens to cease drug abuse and prevent relapse.
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The authors contributed equally to the present review.

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