Identification of genes and gene pathways associated with major depressive disorder by integrative brain analysis of rat and human prefrontal cortex transcriptomes

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Despite moderate heritability estimates, progress in uncovering the molecular substrate underpinning major depressive disorder (MDD) has been slow. In this study, we used prefrontal cortex (PFC) gene expression from a genetic rat model of MDD to inform probe set prioritization in PFC in a human post-mortem study to uncover genes and gene pathways associated with MDD. Gene expression differences between Flinders sensitive (FSL) and Flinders resistant (FRL) rat lines were statistically evaluated using the RankProd, non-parametric algorithm. Top ranking probe sets in the rat study were subsequently used to prioritize orthologous selection in a human PFC in a case–control post-mortem study on MDD from the Stanley Brain Consortium. Candidate genes in the human post-mortem study were then tested against a matched control sample using the RankProd method. A total of 1767 probe sets were found to be significantly dysregulated between human cases and controls at q ≤ 0.001. A total of 898 orthologous probe sets was found on Affymetrix’s HG-U95A chip used in the human study. Correcting for the number of multiple, non-independent tests, 20 probe sets were found to be significantly dysregulated between human cases and controls at q ≤ 0.05. These probe sets tagged the expression profile of 18 human genes (11 upregulated and seven downregulated). Using an integrative rat–human study, a number of convergent genes that may have a role in pathogenesis of MDD were uncovered. Eighty percent of these genes were functionally associated with a key stress response signalling cascade, involving NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), AP-1 (activator protein 1) and ERK/MAPK, which has been systematically associated with MDD, neuroplasticity and neurogenesis.

INTRODUCTION

Major depressive disorder (MDD) is a severe psychiatric disease providing a significant contribution to the global burden of disease.¹,² Endevours to identify factors underlying the molecular basis of MDD have been guided by quantitative studies reporting a substantial genetic contribution to its development.³ However, as with other Axis-I psychiatric disorders, progress in identifying the genetic variation associated with the pathology have been slow.⁴,⁵

One approach for studying the molecular mechanism of MDD in disease-relevant brain regions is by exploring messenger RNA (mRNA) changes in animal models.⁶ The identification of differentially expressed genes can provide early clues into the molecular mechanisms associated with the pathology in humans. However, the exploration of the human brain transcriptome has major limitations, namely the sample limitations, including the requirement of post-mortem brain tissue and confounding factors.⁷ Indeed, several studies have demonstrated the advantage of using animal models of disease to inform human studies by providing a hypothesis-free candidate genes selection with higher prior probability of being involved in the human pathology.⁶,⁷,⁹

In this study, we explored expression differences between Flinders Sensitive or Resistant Lines (FSL/FRL) of rat, which represent one of the most robust genetic models of MDD.¹⁰ Flinders rat have been selectively bred to display a high sensitivity to diisopropyl fluorophosphates and cholinergic agents, mimicking an established neurobiological feature of MDD in humans.¹¹ In addition, these lines have been reported to exhibit a number of other characteristic biological and behavioural features of MDD.¹⁰,¹²

By identifying differentially expressed genes between FSL and FRL lines, it is possible to guide candidate gene selection for subsequent analysis in human post-mortem samples.⁸ A recently published study from the Genome-based Therapeutic Drugs for Depression (GENDEP) consortium previously explored hippocampal expression differences by adopting this approach.⁶ However prefrontal cortex (PFC) expression profiles are now available and existing evidence demonstrates that abnormalities have been repeatedly reported in MDD patients in this brain region.¹³,¹⁴ Therefore, we followed a similar design to investigate gene expression changes in PFC. In this study, identification of differentially expressed genes in the PFC of FSL/FRL was used to inform probe set selection in a comparable human PFC mRNA data set. We hypothesized that a set of genes differentially expressed in the genetic rat MDD model would also be differentially regulated in a human, case–control study on MDD.

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MATERIALS AND METHODS

Design
This study used transcriptomic data derived from the PFC of a rat model of MDD, investigated within the GENDEP project (http://gendep.ion.kcl.ac.uk), to guide candidate gene selection for subsequent analysis within a comparable post-mortem case–control study on MDD from the Stanley Brain Consortium (http://www.stanleyresearch.org). GENDEP is a multi-centre pharmacogenetic project consisting of a series of studies involving humans, animal models and in vitro experiments. GENDEP design was aimed at performing an integrative analysis of key processes to provide insight into the molecular mechanisms underlying MDD and the differential response to antidepressant treatment. One subgroup of rodent studies within the GENDEP project involved comparison of mRNA levels in the PFC of FSL and FRL rat lines, a robust model of ‘endogenous’ depression. Candidate modulated genes identified in the animal model were subsequently validated in human samples. The case–control study of MDD by the Stanley Brain Consortium similarly collected information of key molecular processes within post-mortem tissue derived from participants using exclusion criteria which can be found on the Stanley Brain Consortium website (http://www.stanleyresearch.org).

Animals
This study used 39 adult rats consisting of two strains, 17 FSL and 22 FRL. Rats were bred in Stockholm at the Karolinska Institutet. Animal maintenance and experimental procedures were conducted in accordance to the European Communities Council Directive of 24 November 1986.

Human samples
This study used human samples, which have been made available to researchers worldwide, after being donated to the Stanley Foundation Brain Collection in MD, USA. The data were downloaded from the Gene Expression Omnibus (accession ID: GSE12654; www.ncbi.nlm.nih.gov/geo). Dissection of PFC tissues (Bromdamm’s Area 10) and microarray procedures were carried out by Iwamoto et al. Post-mortem PFCs from individuals diagnosed with MDD were carefully matched with controls. Diagnoses of MDD were in accord with the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. Controls were matched by age, gender, race, pH, post-mortem interval and quality of mRNA extraction. Consistent with the summarized data, the PRadvance function within the RankProd software determines the probability that each biological function assigned to that data set was due to chance alone.

RESULTS

The analysis uncovered a total of 15 genes, 11 significantly upregulated and four significantly downregulated in MDD cases versus control. The list includes several genes previously implicated in depression including the NTRK2, AXL and TAC1 genes. Affymetrix’s arrays are designed to have multiple probe sets tagging the expression of different genes in the 3’UTR region. Different probe sets tagging the expression of the same genes were among the top ranking probe sets further reducing the chance of false positives. The fold change of significant probes ranged from 1.18 to 1.80. As the findings are in human brain, moderate fold changes in gene expression are expected due to large heterogeneity and the relatively sensitive neural environment. Last, we uploaded all 20 probes sets on the IPA software. Nineteen probe sets were mapped to the ingenuity reference database and carried forward for analysis. The top ranking pathway returned by IPA, with a score of 26 and included 12 out of 15 reference molecules uploaded. The majority of the genes uncovered (80%) are significantly associated with same network
centred on the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), MapK and ERK signalling cascade. This neurogenetic-neurotrophic pathway is of particular interest and relevance as it has been extensively associated with MDD and stress response.

**DISCUSSION**

This study has used transcriptomic data derived from the PFC of FSL and FRL rat strains to guide candidate gene selection in human samples generated in the corresponding brain region and subsequently identify molecular dysregulations associated with MDD in the PFC of humans. Analysis of differential gene expression within the PFC of the selectively bred FSL and FRL strains, a robust model of MDD, provided an appropriate number of genes to be then validated in the more relevant human data set. The analysis of differential gene expression in the PFC of individuals diagnosed with MDD or controls, using the candidate gene selection, provided a list of genes and biological pathways associated with MDD.

**Table 1.** Table shows probe ID, gene title, gene symbol, FC (fold change), pfp (percentage of false positives) and P-value for probes significantly associated with MDD

| Probe ID | Gene title | Gene symbol | FC   | pfp   | P-value |
|----------|------------|-------------|------|-------|---------|
| 31525_s_at | Haemoglobin, alpha 1 | HBA1 | 1.80 | 0.0E+00 | <1.00E–06 |
| 32052_at  | Haemoglobin, beta | HBB | 1.67 | 0.0E+00 | <1.00E–06 |
| 31687_s_at | Haemoglobin, beta | HBB | 1.65 | 0.0E+00 | <1.00E–06 |
| 40928_at  | WD repeat and SOCS box containing 1 | WSB1 | 1.35 | 9.2E–03 | <1.00E–06 |
| 38280_s_at | Neurotrophic tyrosine kinase, receptor, type 2 | NTRK2 | 1.28 | 1.1E–02 | 1.00E–04 |
| 1603_g_at  | Protein kinase C, iota | PRKCI | 1.35 | 1.7E–02 | 1.00E–04 |
| 33182_at  | Neurotrophic tyrosine kinase, receptor, type 2 | NTRK2 | 1.27 | 2.5E–02 | 2.00E–04 |
| 31809_at  | Contactin 1 | CNTN1 | 1.22 | 3.1E–02 | 2.00E–04 |
| 36059_at  | Low density lipoprotein-receptor-related protein 4 | LRPR | 1.31 | 3.1E–02 | 3.00E–04 |
| 1602_at   | Protein kinase C, iota | PRKCI | 1.32 | 3.0E–02 | 3.00E–04 |
| 33364_at  | Phosphodiesterase 4D interacting protein | PDE4DIP | 1.22 | 2.7E–02 | 3.00E–04 |
| 32786_at  | jun B proto-oncogene | JUNB | 1.33 | 2.6E–02 | 4.00E–04 |
| 1278_at   | AXL receptor tyrosine kinase | AXL | 1.28 | 3.8E–02 | 6.00E–04 |
| 39827_at  | DNA-damage-inducible transcript 4 | DDIT4 | 1.27 | 3.8E–02 | 6.00E–04 |

Abbreviation: MDD, major depressive disorder.

A number of genes differentially regulated in MDD individuals indicate increased activation of stress and oxidative response pathways in the PFC. Two probes within PKCI (protein kinase C iota) were identified as upregulated in MDD. PKCI encodes a serine/threonine protein kinase, which has been reported to regulate NF-κB activity and neurotrophin-mediated neuronal differentiation and survival via neuronal growth factor. PKCI has been previously associated with MDD in a previous analysis using the same human data set. PKCI has also been determined as upregulated in suicidal individuals, compared with non-suicidal populations, both with mood disorders. These findings support PKCI as a modulator of mood disorders. Another gene associated with MDD involved in the stress response is DDIT4. DDIT4 (DNA-damage-inducible transcript 4), also known as REDD1, is an inhibitor of mTORC1 (mammalian target of rapamycin complex-1) and it is regulated by oxidative stress. Recent findings demonstrated DDIT4 involvement. DDIT4 was activated in the PFC of rats in response to stress, viral-mediated upregulation of DDIT4 in the PFC of rats induced depressive-like and anxiety-like behaviours, and the analysis of different post-mortem MDD samples showed an upregulation of DDIT4 in the PFC of MDD individuals. Our findings are in agreement with the upregulation of DDIT4 in the PFC of MDD patients, highlighting the potential importance of mTORC1 pathways in MDD.

One of the main theories of the pathophysiology of MDD asserts that the exposure to chronic stress alters transcriptional regulation of growth factors and hormones leading to impaired neurogenesis and neuroplasticity. According to this theory, following our findings of altered stress response pathways, genes
involved in neuroplasticity and neurogenesis were also expected to be differentially expressed. Compelling evidence showed an association between dysregulation of neurotrophic pathways, impaired neuronal plasticity and MDD. The neuroplastic functions of the neurotrophic pathway are centred on brain-derived neurotrophic factor (BDNF), its receptor neurotrophic tyrosine receptor kinase type 2 (NTRK2 or TrkB) and the transcription factor cAMP (cyclic adenosine monophosphate) binding protein 1 (CREB1) which regulates both the expression of BDNF and the TrkB gene. In this study, two key genes in the neurotrophic pathway were associated with MDD in the PFC, in agreement with this theory. In our results, the clearest evidence for the dysregulation of neurotrophic pathways in MDD comes from the finding of an upregulation of the NTRK2 gene in MDD patients as shown by two probes. The NTRK2 gene encodes the neurotrophic tyrosine receptor kinase type 2 (TrkB), which binds BDNF as well as other neurotrophins, including NT-4 (neurotrophin-4) and NT-3 (neurotrophin-3). Previous studies have reported that antidepressant treatment leads to an upregulation of BDNF expression and activation of NTRK2 via CREB. Moreover,

Figure 1. Network uncovered using IPA. The network includes 12 out of the 15 genes uploaded on the Ingenuity Pathway Analysis reference database. These genes are functionally related and have a role in the same network centred on NF-κb, MAPK and ERK signalling cascade. This network has previously been implicated in both MDD studies and in studies on response to antidepressant treatment. Genes central to the stress response signalling cascade have been highlighted in red. Genes identified as differentially expressed in MDD individuals have been highlighted: pink indicates upregulation and blue indicates downregulation. IPA, Ingenuity Pathway Analysis; MDD, major depressive disorder; NF-κb, nuclear factor kappa-light-chain-enhancer of activated B cells.
genetic variation within and downregulation of NTRK2 in the PFC has been associated with suicidal ideation and suicide,\textsuperscript{35,36} supporting the role of NTRK2 in depressive symptoms.

Another gene significantly upregulated in MDD patients was PDE4DIP (phosphodiesterase 4D interacting protein). PDE4DIP's function is to compartmentalize PDE4D within the cAMP pathway.\textsuperscript{37} PDE4 proteins degrade cAMP and are important for the regulation of intracellular cAMP concentrations. Antidepressant effects exerted by PDE4 inhibitors through the enhancement of cAMP signalling are mainly attributable to the inhibition of PDE4D.\textsuperscript{38} It is conceivable that our finding of PDE4DIP upregulation in MDD individuals indicates increased compartmentalization of PDE4D leading to altered cAMP availability. This provides a link between PDE4DIP and neurotrophin regulation via CREB1.

The AXL gene, encoding AXL receptor protein-tyrosine kinase (RPTK) was shown in our analysis to be upregulated in MDD individuals. This subfamily of RPTKs has been less well characterized in the brain than the previously discussed neurotrophic receptor tyrosine kinases. The AXL RPTKs acts as the receptor for Gas6 (growth arrest-specific gene-6), which is expressed throughout the adult central nervous system. A study investigating the function of AXL RPTKs in the adult rat brain suggested a role in neuronal survival and growth, and regulating synaptic function and plasticity.\textsuperscript{39} Further research is required to determine the extent to which these RPTKs regulate the molecular mechanisms of depression, however, the AXL gene identified as a biomarker for depressive symptoms in elderly individuals.\textsuperscript{40}

TAC1 (tachykinin, precursor 1), reported as downregulated in MDD individuals, is a complex gene encoding four proteins within the tachykinin hormone family. These four proteins, called substance P, neurokinin A, neuropeptide K and neuropeptide gamma, function as neurotransmitters and neuromodulators. Our finding is supported by a study reporting that Tac1 knockout in mice led to reduced anxiety-like and depression-like behaviours.\textsuperscript{41} In addition, antagonists of the tachykinin receptor, called TACR1, have been investigated in clinical trials for depression with mixed results.\textsuperscript{42} Research of TACR1 antagonists is still under investigation for antidepressant potential. Further research into the protein products of TAC1 and other neurokinins could provide insight into the molecular mechanisms of MDD.

DUSP6 (dual-specificity protein phosphatase 6) was identified in this study, as downregulated in the PFC of MDD individuals. The downregulation of DUSP6 has also been reported in the PFC of individuals with bipolar disorder.\textsuperscript{43} Furthermore, genetic variation within and proximal to DUSP6 has been associated with bipolar disorder and lithium-induced ERK activation,\textsuperscript{44} a mechanism related to neuropsychiatry and neurogenesis,\textsuperscript{45} suggesting a role for DUSP6 in the regulation of mood disorders, neuropsychiatry and neurogenesis.

Another pathway implicated in depression with effects on neuropsychiatry and neurogenesis is under the regulation of the growth factor erythropoietin, both independent and dependent on its role in haematopoiesis.\textsuperscript{46,47} Our results provide some evidence that erythropoietin pathways are altered in MDD individuals with the three most significantly upregulated probe sets representing two genes, HBA and HBB (haemoglobin alpha and beta). This finding is quite interesting as it could be interpreted in a number of ways. It is possible that the upregulation of these haemoglobin genes is indicative of an increased concentration of haemoglobin in the PFC of individuals with MDD. However, another more likely interpretation of these findings includes a negative feedback loop where decreased haemoglobin in the PFC of individuals with MDD initiates an upregulation of haemoglobin genes. For example hypoxia induces an upregulation of Hif1 (hypoxia inducible factor) and subsequently HBA and HBB.\textsuperscript{48,49}

Low levels of systemic haemoglobin have symptoms of high levels of fatigue and lethargy, similar to symptoms seen in some depressed individuals. Low peripheral haemoglobin levels in cancer patients have been reported to correlate with severity of depression,\textsuperscript{50} even when controlling for severity of cancer.\textsuperscript{51} Moreover, it was reported that a low level of haemoglobin was a significant risk factor for post-partum depression even when controlling for confounding variables such as delivery complications and perinatal blood loss.\textsuperscript{52} However, measures of haemoglobin in the brain would be required to establish a connection with depression. One study used near-infrared spectroscopy to measure concentrations of oxygenated haemoglobin, as a parameter for activity, in the PFC of MDD individuals and controls.\textsuperscript{53} This study reported a significant decrease in oxygenated haemoglobin in the PFC of MDD individuals, particularly in Brodmann’s area 10. This finding and our result of upregulated haemoglobin genes in Brodmann’s area 10 suggest low levels of anaemia in the PFC of depressed individuals requiring further research. It is possible that if this anaemia does occur in the brain, it could initiate stress response pathways via hypoxia-induced factors.\textsuperscript{54}

Strengths and limitations
The first limitation to this study is its use of post-mortem tissue for the isolation of human PFC RNA. RNA rapidly degrades and therefore isolation must be carried out carefully to preserve the quality of the sample. In addition, due to the environmental sensitivity of gene expression, the process of death could cause global transcriptomic changes throughout the body creating artefacts in the results. However, without the use of post-mortem tissue, it is not possible to extract RNA from MDD-relevant brain tissue in humans. We have applied an integrative cross-species approach, which helps to overcome these issues by selecting candidate genes from the analysis of the FSL model in which relevant brain tissue is accessible and environmental factors can be controlled. Although relatively stringent thresholds were used throughout the analysis, further replication of findings may be required.

Second, the use of an animal model to study complex behavioural phenotypes in humans is limited, due to the impossibility to reproduce all the complex features of a psychiatric disease in rodents. However animal models have been shown to be an important source of information for the study of depression and antidepressant treatment response in humans.\textsuperscript{5} Third, although we used a cross-validation method by exploring mRNA differences across different studies, verification of findings with other experimental methods such as quantitative PCR would have been desirable; results should be interpreted in light of this limitation.

Last, the human data were derived from just one subsection of the PFC; Brodmann’s area 10, meaning this study was unable to see how gene expression changes associated with MDD varied in other subsections of the PFC.

In summary, the findings highlight the importance of stress response pathways leading to altered regulation of neurogenetic and neurotrophic pathways such as the neurotrophin and neurokinin pathways, supporting previous reports of increased stress response and impaired neuroplasticity underlying the pathophysiology of MDD. Further research on the mechanism by which these molecules alter depressive behaviour could improve prevention, diagnosis and treatment of MDD.

CONFLICT OF INTEREST
The authors declare no conflict of interest.
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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)