

## ORIGINAL ARTICLE

# Gene expression patterns in the hippocampus and amygdala of endogenous depression and chronic stress models

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The etiology of depression is still poorly understood, but two major causative hypotheses have been put forth: the monoamine deficiency and the stress hypotheses of depression. We evaluate these hypotheses using animal models of endogenous depression and chronic stress. The endogenously depressed rat and its control strain were developed by bidirectional selective breeding from the Wistar–Kyoto (WKY) rat, an accepted model of major depressive disorder (MDD). The WKY More Immobile (WMI) substrain shows high immobility/despair-like behavior in the forced swim test (FST), while the control substrain, WKY Less Immobile (WLI), shows no depressive behavior in the FST. Chronic stress responses were investigated by using Brown Norway, Fischer 344, Lewis and WKY, genetically and behaviorally distinct strains of rats. Animals were either not stressed (NS) or exposed to chronic restraint stress (CRS). Genome-wide microarray analyses identified differentially expressed genes in hippocampi and amygdalae of the endogenous depression and the chronic stress models. No significant difference was observed in the expression of monoaminergic transmission-related genes in either model. Furthermore, very few genes showed overlapping changes in the WMI vs WLI and CRS vs NS comparisons, strongly suggesting divergence between endogenous depressive behavior- and chronic stress-related molecular mechanisms. Taken together, these results posit that although chronic stress may induce depressive behavior, its molecular underpinnings differ from those of endogenous depression in animals and possibly in humans, suggesting the need for different treatments. The identification of novel endogenous depression-related and chronic stress response genes suggests that unexplored molecular mechanisms could be targeted for the development of novel therapeutic agents.

*Molecular Psychiatry* (2012) 17, 49–61; doi:10.1038/mp.2010.119; published online 16 November 2010

**Keywords:** animal models; depression; microarray; selective breeding; Wistar Kyoto rat

## Introduction

Major depressive disorder (MDD) is the third most prevalent and costly disease worldwide, and it is projected to become number one by 2030 (WHO, GBD report, 2004). In addition to increasing the risk for suicide, MDD presents frequent comorbidity with other psychiatric disorders, and it has been identified as a risk factor for many illnesses, including obesity, cardiovascular, neurodegenerative and other diseases.<sup>1–4</sup> Understanding the pathophysiology of MDD, and providing better treatments, would im-

prove mental health and quality of life of the largest psychiatric population in the world.<sup>5</sup>

There are two major hypotheses with regard to the etiology of MDD: the monoamine deficiency and stress hypotheses of depression. The monoamine deficiency hypothesis proposes that MDD can be etiologically explained by a deficiency in the monoamine neurotransmitters serotonin, norepinephrine and dopamine. Although this hypothesis is still prominent and is the basis of most antidepressant development to date, current opinion is that monoamine deficiency only partially explains MDD.<sup>6–8</sup> On the basis of observations in MDD, the stress hypothesis relates pathological alterations in the stress-responsive hypothalamic-pituitary-adrenal (HPA) axis to causality of depression.<sup>9–17</sup> Despite a large body of data supporting a role for stress in MDD, the nature and degree of its involvement remains uncertain.<sup>18</sup> Knowing whether stress-induced depression

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Received 6 May 2010; revised 5 October 2010; accepted 11 October 2010; published online 16 November 2010

shares any or all molecular mechanisms with that of endogenous depression may guide the development of different treatment alternatives.

Animal models that reproduce key symptoms of MDD offer a unique opportunity for experimental exploration unfeasible in human studies.<sup>13,14,17,19–22</sup> Selective breeding has been used to generate animal models of depression with extreme traits.<sup>23–28</sup> We used this approach to create two substrains of the Wistar Kyoto (WKY) rat, an accepted model of MDD that replicates many behavioral and physiological endophenotypes present in major depression.<sup>29–32</sup> The two substrains of the WKY, identified as WKY More Immobile (WMI) and WKY Less Immobile (WLI), were generated by bidirectional selective breeding from the WKY based on depressive behavior in the forced swim test (FST) of the naïve, unstressed animals; therefore, WMIs represent an endogenous depression model.<sup>33</sup> The chronic stress model employed four phylogenetically, physiologically and behaviorally different rat strains<sup>34,35</sup> to identify general, strain-independent molecular characteristics of the chronic stress response. We chose a chronic restraint stress (CRS) paradigm that has been shown to increase depression-like behaviors<sup>36–42</sup> and reduce hippocampal volume.<sup>43–45</sup>

The hippocampus and amygdala were selected to investigate gene expression profiles, as these brain regions have been strongly implicated in the cause and consequences of both depression and chronic stress.<sup>46–51</sup> Our goal was to examine both the monoamine and stress hypotheses of depression, the former by identifying the contribution of genes implicated by the monoamine hypothesis to the two depression model expression profiles, and the latter by defining the overlap between the expression profiles of endogenous depression and chronic stress response. Our present results found no evidence for the involvement of monoamine neurotransmission-related genes in the expression profile of the endogenous depression model and found divergent expression profiles in endogenous depression and chronic stress models. However, novel target genes and pathways have emerged from this study, which have the potential to advance our knowledge with regard to the etiology of depression and its treatment.

## Methods

### *Animal models*

All procedures were approved by the Institutional Animal Care and Use Committee of Northwestern University. The WMI-WLI selective breeding commenced as described previously.<sup>33</sup> Animals showing the most extreme FST behavior within each line were selected for breeding, specifically avoiding sibling mating until the G5 generation. Adult WMI and WLI male animals from the 13th generation of selective breeding were employed in this study. The experimental design included daily administration of desipramine (10 mg kg<sup>-1</sup>) or saline, subcutaneously

for 14 days, to both WMI and WLI animals ( $n=9$  per treatment per strain). Animals were killed by fast decapitation on the 15th day immediately after removal from the home cage.

For the CRS experiment, adult male Fischer 344 (F344), Brown-Norway (BN), Lewis (LEW) and WKY rats were obtained from Harlan Laboratories (Indianapolis, IN, USA) ( $n=9$  per strain per treatment). Rats were either exposed to CRS in a breathable decapicone for 2 h per day for a 2-week period or remained in their home cage (no stress, NS). Body weight was monitored throughout the experiment. On the 15th day, both groups of rats were tested in the elevated plus maze test in parallel and killed by decapitation immediately following the 5-min test. Animals were ~100 days old at the time of sample collections. Blood samples were collected for the determination of plasma corticosterone (CORT) levels and adrenal weight was also determined. The elevated plus maze test of anxiety was used as a confirmation of strain differences in anxiety measures basally (NS group) and after CRS: the results are not shown since the purpose of this study is to identify chronic stress-induced changes in gene expression independently of strain effects.

### *Behavioral testing*

FST was performed as described previously.<sup>30,52</sup> Briefly, on day 1, rats were individually placed in the water tank (water temperature 22–24 °C) for 15 min. After 24 h, rats were once again placed in the tank for a 5-min test session. The test session was videotaped and scored by a trained observer, using the scoring system developed by Detke *et al.*<sup>53</sup> The open-field test was performed as described previously,<sup>54</sup> but the animals' movements were followed and analyzed by the TSE Videomot 2 version 5.75 software.

### *Radioimmunoassay for plasma CORT*

Assays were carried out in duplicate, as described previously,<sup>29</sup> using the CORT RIA kit (MP Biomedicals, Solon, OH, USA). The assay sensitivity was 2–4 pg per tube. The intra- and interassay coefficients of variation were 3.5 and 8%, respectively.

### *Microarray experiments*

Brain regions were dissected immediately after decapitation, as described previously,<sup>55</sup> and stored at –80 °C in RNAlater (Ambion, Austin, TX, USA). Individual hippocampi and amygdalae were homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA), and RNA was isolated following the manufacturer's protocol. All RNA samples were treated with DNase1 (Qiagen, Valencia, CA, USA) according to the manufacturer's methods.

Total RNA isolated from WMI and WLI brain regions was reverse-transcribed, and double stranded cDNA was synthesized with the GeneChip<sup>®</sup> Expression 3-Amplification One-cycle kit (Affymetrix, Santa Clara, CA, USA). In an *in vitro* reaction with T7 RNA

polymerase, the cDNA was linearly amplified and labeled with biotinylated nucleotides (Affymetrix). Ten micrograms of biotin-labeled and fragmented cRNA was then hybridized onto Rat Genome 230 2.0 GeneChip arrays (Affymetrix).

Total RNA from the chronic stress experiment was reverse transcribed, followed by second-strand cDNA synthesis. For each sample, an *in vitro* transcription reaction was carried out incorporating biotinylated nucleotides according to the manufacturer's protocol for Illumina® Totalprep RNA amplification kit (Ambion). Biotin-labeled cRNA, 1.5 µg, was then hybridized onto RatRef-12 Expression BeadChips (Illumina, San Diego, CA, USA).

#### Statistical analysis for WMI-WLI experiment

Probe intensity data from Rat 230v2 Affymetrix GeneChip arrays were read into the R software environment (<http://www.R-project.org>) directly from CEL files using the R/affy package.<sup>56</sup> Affymetrix data was normalized using the robust multiarray average method for probe set data.<sup>57</sup> Data quality was assessed using histograms of signal intensities, scatter plots and hierarchical clustering of samples.

Analysis of variance (ANOVA) methods, performed with the R/maanova package,<sup>58,59</sup> were used to statistically resolve gene expression differences. The experimental design included chronic administration of desipramine or saline to both WMI and WLI animals, of which three animals per strain per treatment were randomly selected for the microarray analyses. As we were only interested in the strain effect in this experiment, treatment (desipramine or saline) was a covariate. For each probe set  $g$ , strain  $i$ , covariate  $j$  and replicate  $k$ , a linear model for the log-transformed expression measure,  $y_{ijk}$ , can be formulated as a sum of components that contribute to the overall intensity value:

$$y_{ijk} = \mu_g + \alpha_{ig} + \beta_{jg} + (\alpha\beta)_{ijg} + \varepsilon_{ijk} \quad (1)$$

where  $\mu_g$  is the mean intensity over all 12 samples,  $\alpha_{ig}$  is the effect of strain  $i$  ( $i=1, 2$ ),  $\beta_{jg}$  is the additive effect of covariate  $j$  ( $j=1, 2$ ),  $(\alpha\beta)_{ijg}$  is the interaction effect of strain  $i$ , covariate  $j$  and probe set  $g$  and  $\varepsilon_{ijk}$  is the residual error for strain  $i$ , covariate  $j$ , replicate  $k$  and probe set  $g$ , respectively. To identify transcripts with a high probability of being differentially expressed between strains, statistical tests for the null hypothesis  $H_0: \alpha_{ig}=0$  vs the alternative  $H_0: \alpha_{ig} \neq 0$  were performed. We excluded transcripts in which the strain effect was not consistent between different covariate levels, as identified by a statistical test for the null hypothesis  $H_0: (\alpha\beta)_{ijg}=0$  vs the alternative  $H_0: (\alpha\beta)_{ijg} \neq 0$ . Both these tests were performed using Fs, a modified F statistic incorporating shrinkage estimates of residual variance.<sup>60</sup>  $P$ -values were calculated by permuting model residuals 1000 times. Unless otherwise noted, transcripts with differences between strains were identified as those with  $P$ -values, or estimated false-positive rates, less than 0.01 for the hypothesis  $H_0: \alpha_{ig}=0$  and greater than 0.01 for the

hypothesis  $H_0: (\alpha\beta)_{ijg}=0$ . As an adjustment for multiple testing, we used the  $q$ -value transformation of the  $P$ -values to estimate false discovery rate.<sup>61</sup> To relate probe sets to genes, probe set IDs were mapped to symbols using NetAffx (<http://www.affymetrix.com/analysis/netaffx/>). When reporting fold changes, we consider WLI to be the reference strain. If the measured expression level is higher in the WMI strain than in the WLI strain, the fold change is positive, and if it is lower in WMI than in WLI, the fold change is negative.

For each experiment, we also applied models of combined data from both tissues to measure the differences in transcript abundance between tissue. We used the model

$$y_{ijklg} = \mu_{mg} + \alpha_{ig} + \beta_{jg} + (\alpha\beta)_{ijg} + \gamma_{kg} + \varepsilon_{ijklg} \quad (2)$$

which is equivalent to model (1), except that there is an additional variable ( $\gamma_{kg}$ ) for tissue with indices  $k=1,2$  for the amygdala and hippocampus. Because we applied the normalization separately by tissue, we mean centered the normalized samples before applying this model.

#### Statistical analysis for stress experiment

Probe intensity data from Illumina Chip arrays were read directly into the R software environment from bead summary files produced by BeadStudio using the R/beadarray package.<sup>62</sup> Quantile normalization was applied to the Illumina bead summary data using the R/preprocessCore package.<sup>63</sup> Data quality was assessed as described for the WMI-WLI experiment.

Analysis of variance methods were similarly applied for this experiment. Twelve rats were exposed to chronic stress conditions, and 12 rats were not exposed to chronic stress. The animals for each stress condition consisted of three rats each from four different strains (BN-SS, F344, LEW and WKY). The linear model of equation (1) was applied for each probe set  $g$ . In this case,  $\alpha_{ig}$  is the effect of condition  $i$  ( $i=1, 2$ ),  $\beta_{jg}$  is the additive effect of strain  $j$  ( $j=1, 2, 3, 4$ ), which is a covariate. To identify transcripts exhibiting consistent differential expression owing to condition across strains, we performed and applied statistical tests for the null hypotheses  $H_0: \alpha_{ig}=0$  vs  $H_0: \alpha_{ig} \neq 0$  and  $H_0: (\alpha\beta)_{ijg}=0$  vs  $H_0: (\alpha\beta)_{ijg} \neq 0$  that are analogous to those described above for the WLI-WMI comparison. Probe set IDs were mapped to symbols using Illumina's annotation resource ([http://www.illumina.com/support/annotation\\_files.ilmn](http://www.illumina.com/support/annotation_files.ilmn)). When reporting fold changes, we consider the NS to be the reference condition. If the measured expression in the CRS condition is greater than in the NS condition, the fold change is positive. If it is less under CRS than under NS, the fold change is negative. To facilitate across-experiment comparison by gene, probes were mapped to gene symbols using current probe annotation files provided by the array manufacturers. The suffixes '\_predicted' and '\_mapped' were truncated from the Illumina symbol assignments. Where there were multiple

probes for the same symbol, probes with the largest *F* statistic for the relevant hypothesis test were chosen to give a one-to-one mapping across experiments for 10 112 genes. The linear model of equation (2) was applied analogously for this experiment to obtain the distribution of estimated tissue effects.

#### Real-time RT-PCR

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was used to confirm microarray results for a subset of randomly selected genes from those fulfilling the criteria of significant ( $P < 0.01$ ) expression differences. Reverse transcription for 2  $\mu\text{g}$  of each sample was performed with Invitrogen's Superscript<sup>®</sup> III First-Strand kit (18080-051) according to the manufacturer's protocol. Primers were designed to amplify 80–150 bp regions and to contain a maximum amount of microarray probe sequence using default settings of ABI's Primer Express software (version 3.0, PE Applied Biosystems, Carlsbad, CA, USA). Primer sequences are listed in Supplementary Table S1. Forty nanograms of cDNA was amplified in 20  $\mu\text{l}$  reactions ( $1 \times$  SYBR green reaction mix (ABI, Carlsbad, CA, USA), 250  $\mu\text{M}$  primers) in the ABI 7900HT PCR machine using the relative quantification ( $-\text{ddCt}$ ) method, with 18s RNA as the internal control.

## Results

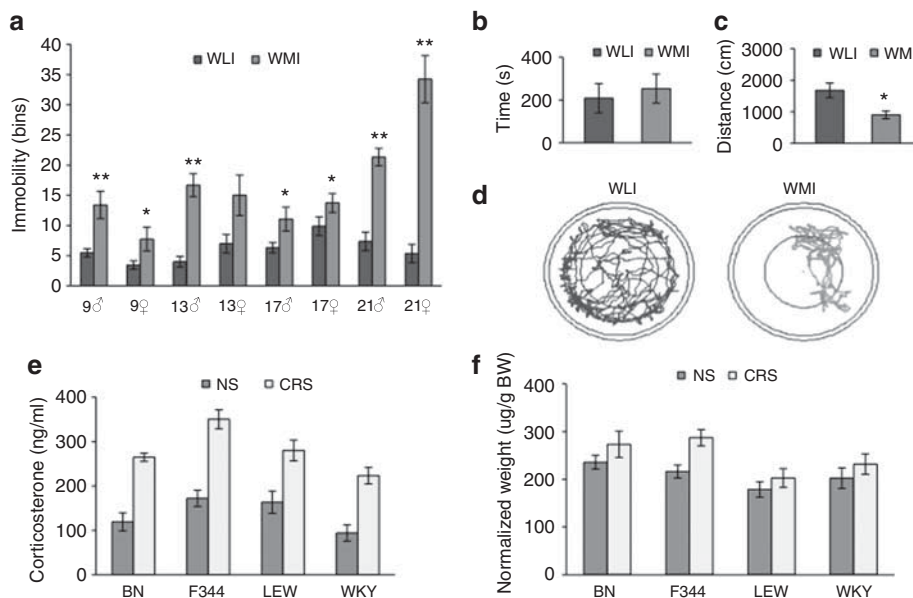
### Selective breeding and characterization of WMI and WLI strains

The WMI and WLI rat strains consistently maintained significant, dichotomous FST phenotypes throughout

21 generations of selective breeding (Figure 1a), with WMIs always exhibiting greater immobility scores than WLIs. In the open-field test of exploration/anxiety, WMI and WLI male animals showed similar level of exploration of the inner circle (Figure 1b), suggesting no differences in anxiety-related behaviors. In contrast, WMIs explored the arena significantly less than WLIs (Figure 1c), and the activity traces (Figure 1d) illustrate freezing-like behavior exhibited by the WMIs, a behavioral pattern very similarly to psychomotor retardation. There were no differences in the two strains' basal plasma thyroxine and CORT levels or their CORT responses to acute restraint stress (Supplementary Table S2). Furthermore, no additional behavioral differences were observed between the two strains, including anxiety-like behaviors as assessed by the elevated plus maze and defensive burying tests, confirming the results of the open-field test, and spatial learning and memory as measured in the Morris water maze (Supplementary Table S2). These data verify that the behavioral differences between the two substrains of WKYs are not fear or anxiety driven, but rather related to depressive state.

### CRS decreased body weight gain, increased plasma CORT levels and adrenal weight

Genetic polymorphisms between the strains range from 25.9% between F344 and LEW to 66% between BN and WKY, representing a substantial interstrain variation. However, CRS affected all strain of rats. Specifically, CRS resulted in a lower body weight gain compared with NS rats ( $F[1,65] = 212.9$ ,  $P < 0.001$ ), but



**Figure 1** The endogenous depression model, the Wistar–Kyoto More Immobile (WMI) strain, shows depressive behavior not linked to fear/anxiety (a–d). Chronic restraint stress (CRS) increases adrenocortical function consistently in all four strains (e and f). (a) In the forced swim test (FST), immobility scores of the WMI and WKY Less Immobile (WLI) animals differ significantly across generations. (b) Time spent in the inner circle of the open-field test, (c) total distance traveled and (d) movement traces of representative WMI and WLI animals. (e) Plasma corticosterone levels were consistently elevated after CRS in all four strains and (f) adrenal weights were consistently greater in the CRS group of all strains. \* $P < 0.01$ , \*\* $P < 0.001$ .

there was no weight loss in response to the CRS paradigm (body weight on day 1:  $239 \pm 5$  g; on day 14 of CRS:  $244 \pm 5$  g). Plasma CORT levels were elevated in all rats exposed to the chronic stress procedure compared with those not subjected to stress (Figure 1e; strain:  $F[1,65]=105.37$ ,  $P<0.001$ ) and those subjected to stress ( $F[3,65]=6.75$ ,  $P<0.001$ ; strain, stress interaction NS). As expected, chronic stress increased adrenal weight in all four rat strains (Figure 1f; strain:  $F[3,41]=1.34$ , NS; stress:  $F[1,41]=4.77$ ,  $P=0.035$ ). Taken together, these data established that all CRS rats were undergoing expected physiological changes associated with CRS exposure.

#### *Differential gene expression profile in the amygdala and hippocampus of WMI and WLI male rats*

Six hundred and thirty-eight genes in the amygdala and 463 in the hippocampus were differentially expressed between the WMI and WLI animals ( $P<0.01$ ). Genes that additionally had fold changes above 1.4 (40% increase or decrease) are listed in Table 1. The complete data set is shown in Supplementary Tables S3 (amygdala) and S4 (hippocampus). Twenty-seven genes were differentially expressed in both brain regions between WMI and WLI in the same direction.

#### *Gene expression changes in response to chronic stress in the amygdala and hippocampus of four strains of male rats*

To eliminate strain-specific effects in our selection attributable to differences in stress reactivity or potential cognitive differences between strains, genes were selected based on consistent, significant expression level differences in all four strains. We found 125 genes in the amygdala and 126 genes in the hippocampus that were differentially expressed between the CRS and NS conditions ( $P<0.01$ ) (Supplementary Tables S5 and S6). From these, the 10 amygdalar and hippocampal genes with absolute fold changes above 1.4 are shown in Table 1. Twelve of the genes showed the same directional changes in both brain regions.

#### *Minimal overlap between depression and stress response*

Both the WMI-WLI and CRS-NS gene sets were examined for brain region-specific overlap using all known aliases to account for possible differences between microarray platform target nomenclatures. Only two genes in the amygdala (*Kcnj14* and *Mpr19*) and four in the hippocampus (*Chi3l1*, collagen type I alpha 1 (*Col1a1*), matrix metalloproteinase 14 (*Mmp14*) and *RGD1305680*) showed differential expression in both the chronic stress response and endogenous depression comparisons. On the basis of the stress hypothesis of depression, transcripts with lowered expression levels in WMI animals should show similarly decreased expression in CRS animals, and increased expression in WMIs should correspond to increased expression in CRS. When we

examined the directionality of fold changes for the six overlapping genes, only three genes (*Col1a1*, *Mmp14* and *RGD1305680*), all in hippocampal tissue, showed these hypothesized directional changes. This provides suggestive evidence that the molecular signature of chronic stress and endogenous depressive behavior in these brain regions are not similar. However, since comparison of overlap between small sets of selected genes can be sensitive to thresholding, we also compared the differences between model effects for CRS and NS with the differences for WMI and WLI.

Expression differences in both models were relatively small (Figures 2a and b). If there is an underlying relationship between expression differences in response to CRS and endogenous depressive behavior for many genes, we should see significant positive correlation between the differences in gene expression across the two experiments. In particular, the differences between WMI and WLI model effects and differences between CRS and NS model effects ( $(\alpha_{2g}-\alpha_{1g})$  in equation (1)) should show positive correlation. The Spearman's correlation ( $\rho$ ) for these comparisons of differences were not significantly different than zero in either tissue ( $\rho=-0.12$ , amygdala;  $\rho=-0.02$ , hippocampus; Figures 2a and b). This lack of observed correlation could be explained by the different platforms or by the chronologies of the experiments. To address this concern, equation (2), which combined data of both brain regions and estimates the tissue effects, was employed. The tissue (interbrain region) effects showed significant positive correlation between experiments ( $\rho=0.48$ ,  $P<0.001$ ; Figure 2c). We also measured correlation for the subset of genes for which the magnitude of tissue effects were comparable to the CRS-NS and WMI-WLI effects, specifically those having a magnitude below 0.4 on both platforms. Within this subset, we still found significant positive correlation ( $\rho=0.31$ ,  $P<0.001$ ). We conclude that we should have observed correlation between the CRS-NS and WMI-WLI effects if it were present despite the platform differences. This suggests that, on a global scale, the effects of chronic stress and endogenous depression on the amygdalar and hippocampal transcriptome are dissimilar.

#### *Confirmation of the expression differences by real-time RT-PCR*

We carried out quantitative RT-PCR confirmation of the microarray data using total RNA samples from both experiments and primer pairs described in Supplementary Table S1. Pearson's correlation of fold change from the Affymetrix microarray for 16 genes with relative quantification ratios from qPCR (RQ ratio) showed significant concurrence of data ( $r=0.720$ ,  $P=0.002$ ; Figure 3a). Pearson's correlation of fold change from the Illumina microarray for nine genes (three were confirmed in both brain regions) with relative quantification ratios from qPCR showed similarly significant correlation ( $r=0.725$ ,  $P=0.008$ ;

**Table 1** Differentially expressed depression genes (top) and stress genes (bottom) in the amygdala and hippocampus

Probe set	Gene name	Rat/human symbol	Cytoband	Fold change	P-value
<i>Amygdala</i>					
1370215_at	Complement component 1, q subcomponent, beta polypeptide	C1qb/C1QB	5q36	-1.60	9.96E-03
1376198_at	Adipocyte-specific adhesion molecule	Asam/ASAM	8q22	-1.60	9.55E-06
1380497_at	Ash1 (absent, small or homeotic)-like (Drosophila)	Ash1/ASH1L	2q34	1.58	1.84E-04
1390835_at	Solute carrier family 47, member 1	Slc47a1/ SLC47A1	10q22- q23	-1.55	5.16E-04
1387029_at	Complement factor H	Cfh/CFH	13q13	-1.54	2.26E-03
1378518_at	Ewing sarcoma breakpoint region 1	Ewsr1/EWSR1	14q21	1.54	8.50E-05
1394940_at	Family with sequence similarity 46, member A	Fam46a/ FAM46A	8q31	-1.53	3.78E-03
1398522_at	Ankyrin repeat and LEM domain containing 2	Ankle2/ ANKLE2	12q16	1.50	8.22E-06
1379917_at	Adaptor-related protein complex 3, sigma 1 subunit	Ap3s1 /AP3S1	18q11	-1.49	3.11E-03
1379264_at	Zinc- and ring finger 1	Znrf1/ZNRF1	4q24	-1.49	7.88E-04
1376175_at	Glioblastoma amplified sequence	Gbas/GBAS	12q13	1.48	1.47E-03
1394964_at	BAT2 domain containing 1	Bat2d1/BAT2D1	13q22	1.48	8.48E-06
1370989_at	Ret proto-oncogene	Ret/RET	4q42	-1.47	1.15E-03
1367749_at	Lumican	Lum/LUM	7q13	-1.46	5.74E-03
1394814_at	Translocated promoter region	Tpr/TPR	13q21	1.46	2.37E-04
1383997_at	Mitogen-activated protein kinase 1	Mapk1/MAPK1	11q23	-1.45	3.46E-04
1391524_at	Similar to WD repeat domain 11 protein	RGD1564964/ PHIP	8q31	1.44	5.17E-05
1369742_at	Hect (homologous to the E6-AP (UBE3A) carboxyl-terminus) domain and RCC1 (CHC1)-like domain (RLD) 1	Herc1/HERC1	8q24	1.44	4.21E-04
1385101_a_at	Coiled-coil domain containing 127	Ccdc127/ CCDC127	1p11	1.43	9.94E-05
1391788_at	Sin3-associated polypeptide, 18 kDa	Sap18/SAP18	15p12	1.43	6.33E-04
1378520_at	B-cell CLL/lymphoma 11B (zinc-finger protein)	Bcl11b/BCL11B	6q32	1.42	2.68E-04
1390112_at	EGF-containing fibulin-like extracellular matrix protein 1	Efemp1/ EFEMP1	14q22	-1.42	2.45E-04
1384000_at	SRY (sex determining region Y)-box 4	Sox4/SOX4	17p12	1.42	6.48E-03
1370891_at	Cd48 molecule	Cd48/CD48	13q24	-1.41	8.88E-03
1381829_at	Zinc-finger protein 318	Zfp318/ZNF318	9q12	1.41	3.40E-03
1370122_at	RAB27B, member RAS oncogene family	Rab27b/ RAB27B	18q12.1	1.40	6.89E-04
1380569_at	Ring finger protein 41	Rnf41/RNF41	7q11	-1.40	7.40E-05
<i>Hippocampus</i>					
1392948_at	Chloride intracellular channel 6	Clic6/CLIC6	11q11	-3.35	4.25E-05
1379281_at	Sclerostin domain containing 1	Sostdc1/ SOSTDC1	6q16	-3.30	2.20E-05
1374320_at	Coagulation factor V (proaccelerin, labile factor)	F5/F5	13q22	-3.12	1.04E-04
1378365_at	Solute carrier family 4, sodium bicarbonate cotransporter, member 5	Slc4a5/SLC4A5	4q34	-2.83	1.39E-04
1370384_a_at	Prolactin receptor	Prlr/PRLR	2q16	-2.61	2.09E-04
1367598_at	Transthyretin	Ttr/TTR	18p	-2.60	5.93E-03
1377434_at	Membrane frizzled-related protein	Mfrp/MFRP	8q22	-2.55	2.20E-04
1375465_at	Orthodenticle homolog 2 (Drosophila)	Otx2/OTX2	15p14	-2.52	4.78E-05
1376944_at	Prolactin receptor	Prlr/PRLR	2q16	-2.25	4.54E-04
1382083_at	Coagulation factor C homolog, cochlin (Limulus polyphemus)	Coch/COCH	6q22	-1.80	8.12E-06
1368606_at	Solute carrier organic anion transporter family, member 1a5	Slco1a5/ SLCO1A2	4q44	-1.79	1.01E-03
1372299_at	Cyclin-dependent kinase inhibitor 1C (P57)	Cdkn1c/ CDKN1C	1q42	-1.77	2.67E-05
1367700_at	Fibromodulin	Fmod/FMOD	13q13	-1.70	1.16E-05
1371849_at	5'-Nucleotidase domain containing 2	Nt5dc2/ NT5DC2	16p16	-1.63	2.72E-04
1369625_at	Aquaporin 1	Aqp1/AQP1	4q24	-1.61	1.29E-03
1367682_at	Midkine	Mdk/MDK	3q24	-1.61	5.17E-04
1368536_at	Ectonucleotide pyrophosphatase/phosphodiesterase 2	Enpp2/ENPP2	7q31	-1.60	3.55E-04
1391211_at	Atpase, class VI, type 11C	Atp11c/ATP11C	Xq36	-1.58	1.36E-03

**Table 1** Continued

Probe set	Gene name	Rat/human symbol	Cytoband	Fold change	P-value
1376728_at	Hypothetical RNA binding protein RGD1359713	RGD1359713/ RBM47	14p11	-1.55	1.24E-03
1375026_at	Calmodulin-like 4	Calml4/ CALML4	8q24	-1.54	1.40E-03
1387791_at	Angiotensin I-converting enzyme (peptidyl-dipeptidase A) 1	Ace/ACE	10q32.1	-1.49	5.35E-03
1368046_at	Solute carrier family 31 (copper transporters), member 1	Slc31a1/ SLC31A1	5q24	-1.49	3.36E-03
1370068_at	Phospholipase A2, group V	Pla2g5/PLA2G5	5q36	-1.48	3.94E-03
1376285_at	GULP, engulfment adaptor PTB domain containing 1	Gulp1/GULP1	9q22	-1.48	6.20E-03
1386770_x_at	Potassium voltage-gated channel, Isk-related subfamily, gene 2	Kcne2/KCNE2	11q11	-1.48	1.69E-03
1374139_at	Cerebellar degeneration-related 2	Cdr2/CDR2	1q35-q36	-1.46	5.40E-04
1368202_a_at	Disabled homolog 2, mitogen-responsive phosphoprotein ( <i>Drosophila</i> )	Dab2/DAB2	2q16	-1.44	2.68E-05
1369798_at	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, beta 2 polypeptide	Atp1b2/ATP1B2	10q24	-1.42	5.24E-03
<i>Amygdala</i>					
4610273	Hemoglobin, beta	MGC72973/HBB	1q32	-1.61	1.91E-03
3140088	Ribosomal protein L30	Rpl30/RPL30	7q22	-1.58	2.07E-03
6650278	Hemoglobin alpha, adult chain 2	Hba-a2/HBA1	10q12	-1.57	1.93E-06
4210725	Transthyretin (prealbumin, amyloidosis type I)	Ttr/TTR	18p12	-1.55	3.47E-03
2510524	Hemoglobin, beta	Hbb/HBB	1q32	-1.53	1.93E-06
<i>Hippocampus</i>					
6650278	Hemoglobin alpha, adult chain 2	Hba-a2/HBA1	10q12	-1.80	2.90E-06
4780059	Insulin-like growth factor 2 (somatomedin A)	Igf2/IGF2	1q41	-1.79	7.16E-05
2510524	Hemoglobin, beta	Hbb/HBB	1q32	-1.75	4.84E-06
4610273	Hemoglobin, beta	MGC72973/HBB	1q32	-1.64	9.67E-05
3840156	Collagen, type I, alpha 2	Col1a2/COL1A2	4q13	-1.46	5.32E-03

Figure 3b). Positive and negative fold changes are defined in Methods.

When we examined the validated transcript fold change in the microarrays in relation to their respective *P*-value, we found no significant correlation ( $r=0.113$ ,  $P>0.05$ ; Figure 3c). These data confirm that significance and fold change criteria cannot be used interchangeably, but that expression changes can be confirmed by real-time RT-PCR, even if the fold change is small.

#### *The molecular pathways of depression and stress response genes*

The lists of hippocampal and amygdalar genes with significant expression differences were combined into single gene lists for each of our two models, and the rat gene symbols were converted to their human orthologs to access a larger body of literature. These master ortholog lists were subjected to functional annotation using the PANTHER Classification System.<sup>64</sup> In addition, we used the PANTHER binomial statistics tool to compare our annotated ortholog lists to the NCBI *Homo sapiens* reference gene list to determine significant over- or under-representation of molecular function, biological process or pathway classification terms.<sup>65</sup>

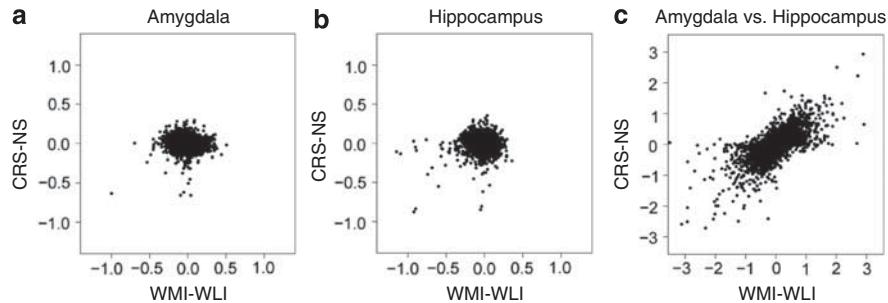
Table 2 shows significantly over- and under-represented terms from both gene sets. A group of

genes identified to belong to the integrin signaling pathway (including *Araf*, *Arf1*, *Arf4*, *Arhgap10*, *Col12a1*, *Col15a1*, *Col1a1*, *Col4a1*, *Col5a2*, *Col6a6*, *Crkl*, *Elmo1*, *Fnlb*, *Grb2*, *Itga11*, *Itga6*, *Itgae*, *Itgb6*, *Itgb8*, *Itgbl1*, *Mapk1*, *Pik3r1*, *Pitk2b*, *Rac1*, *Rhoa* and *Tln2*) were most overly represented in the WMI-WLI comparison. From the CRS-NS comparison, the Huntington disease pathway showed the only significantly clustered group of genes (*Capn9*, *Gapdh*, *Rhog*, *Tnfaip8*, *Tnfaip8l3* and tumor suppressor protein 53).

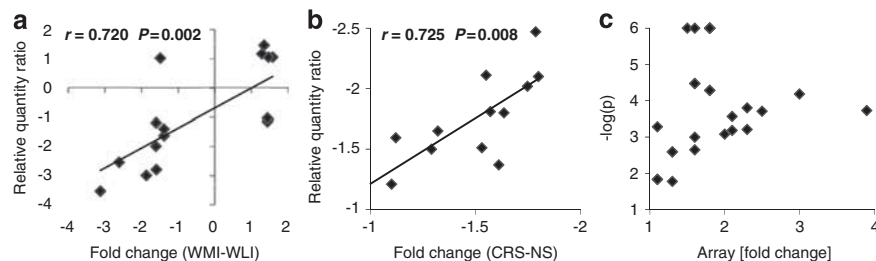
The biological process terms 'Cellular Process', 'Cell Communication' and 'Signal Transduction' were significantly over-represented in both the CRS-NS and the WMI-WLI gene lists. It is of further interest that metabolic process terms are the most over-represented in the WMI-WLI comparison, while cellular process terms, mainly immune system-related, are over-represented in the CRS-NS gene list. There was no overlap in over- or under-represented molecular function terms between the stress response and endogenous depression gene lists.

#### Discussion

This study identified novel molecular signatures of chronic stress and of endogenous depressive behavior in animal models. However, absence of



**Figure 2** An across-experiment, gene-to-gene mapping was created for 10 112 gene symbols. Scatter plots are shown for observed effects obtained from the chronic restraint stress-no stress (CRS-NS) microarray experiment (vertical axis) and the Wistar–Kyoto More Immobile-WKY Less Immobile (WMI-WLI) microarray experiment (horizontal axis) by applying the linear models of Equation (1) and (2). The statistics are differences in condition effects,  $\alpha_{2g}-\alpha_{1g}$ , for the amygdala (a) and hippocampus (b), and differences in brain region-related effects,  $\gamma_{2g}-\gamma_{1g}$  (c).



**Figure 3** (a) Validation of genes differentially expressed in Wistar–Kyoto More Immobile (WMI) and WKY Less Immobile (WLI) hippocampi or amygdala ( $n = 6$  per strain) by real-time reverse transcription-polymerase chain reaction (RT-PCR). The correlation between fold change in the Affymetrix microarray experiment and real-time RT-PCR determination of relative quantification ratios are shown (Pearson's correlation,  $r = 0.720$ ,  $P = 0.002$ ). (b) Validation of genes differentially expressed in chronic restraint stress (CRS) vs no stress (NS) ( $n = 12$  per treatment) hippocampi or amygdala by real-time RT-PCR. The correlation between fold change in the Illumina array experiment and real-time RT-PCR determination of relative quantification ratios are shown (Pearson's correlation,  $r = 0.725$ ,  $P = 0.008$ ). (c) Lack of correlation between absolute fold change and significant  $P$ -value ( $-\log P$ ) from the microarray analyses for genes with expression changes validated by real-time RT-PCR.

direct association between the gene expression profiles suggests that independent molecular mechanisms regulate these two states.

Among genes showing significantly altered transcription levels in the amygdala and hippocampus in the endogenous depression model, there was a complete omission of serotonergic, adrenergic and dopaminergic neurotransmission-related genes. This finding, although surprising, is congruent with gene expression studies of human postmortem MDD brains.<sup>66–68</sup> We did find that multiple newer antidepressant targets, including *Grm5*,<sup>69</sup> and a number of phosphodiesterases,<sup>70</sup> were differentially expressed between WMIs and WLIs. In addition to these previously described targets, we identified several novel depressive behavior-related genes and pathways. Physiologically intriguing findings include the decreased mRNA levels of prolactin receptor and angiotensin I-converting enzyme in the WMI hippocampus; these genes have known peripheral function, but they have not been associated with depressive behavior as yet. Other intriguing examples of novel implications for endogenous depression include the

solute carrier family 4, sodium bicarbonate cotransporter and member 5 gene (*Slc4a5*), which has recently been associated with metabolic phenotypes.<sup>71</sup> Interestingly, metabolic processes were the most over-represented biological process terms in the WMI-WLI gene ontology. The integrin signaling pathway, which was over-represented in the endogenous depression gene set, contains different members of the collagen family, some of which are also implicated in the chronic stress response. Finally, mRNA levels of dehydrololichyl diphosphate synthase are increased in both the hippocampus and the amygdala of WMI. Dehydrololichyl diphosphate synthase is responsible for synthesizing dolichol, which accumulates in the neuropathological human brain.<sup>72</sup>

The set of genes whose hippocampal or amygdalar expression patterns were altered by chronic stress in all four rat strains represent a generalizable molecular response to chronic stress. Many of these genes have shown matching directional changes by unpredictable chronic mild stress, and these include: *Col1a1*, tissue plasminogen activator, insulin-like growth factor binding protein 2, amyloid beta precursor



**Table 2** Over- and under-represented gene ontology (GO) groups determined by the Panther software analysis

	NCBI (19 911) <sup>a</sup>	STR (206) <sup>b</sup>	EXP	+/-	P-value	DEP (1065) <sup>c</sup>	EXP	+/-	P-value
<i>Pathway</i>									
Huntington disease	167	6	1.73	+	<b>8.30E-03</b>	10	9	+	4.04E-01
Integrin signaling pathway	189	3	1.96	+	3.11E-01	26	10	+	<b>1.88E-05</b>
Ras pathway	79	0	0.82	-	4.41E-01	14	4	+	<b>1.28E-04</b>
Heterotrimeric G-protein signaling pathway- Gq alpha- and Go alpha-mediated pathway	134	0	1.39	-	2.49E-01	16	7	+	<b>2.91E-03</b>
Angiogenesis	191	0	1.98	-	1.37E-01	19	10	+	<b>8.55E-03</b>
Axon guidance mediated by semaphorins	43	1	0.44	+	3.59E-01	7	2	+	<b>9.28E-03</b>
<i>Biological process</i>									
Primary metabolic process	7959	95	82.34	+	4.26E-02	533	426	+	<b>1.88E-11</b>
Metabolic process	8276	101	85.62	+	1.82E-02	548	443	+	<b>4.98E-11</b>
Cellular process	6309	83	65.27	+	<b>5.60E-03</b>	433	337	+	<b>4.35E-10</b>
Cell communication	4408	65	45.61	+	<b>1.13E-03</b>	313	236	+	<b>2.13E-08</b>
Protein metabolic process	3247	41	33.59	+	9.86E-02	240	174	+	<b>8.43E-08</b>
Signal transduction	4234	61	43.81	+	<b>3.02E-03</b>	296	226	+	<b>2.73E-07</b>
Transport	2857	42	29.56	+	1.13E-02	213	153	+	<b>3.10E-07</b>
Neurological system process	1995	29	20.64	+	3.89E-02	152	107	+	<b>7.07E-06</b>
Vesicle-mediated transport	1160	13	12	+	4.24E-01	98	62	+	<b>7.86E-06</b>
Intracellular signaling cascade	1609	17	16.65	+	5.01E-01	127	86	+	<b>9.00E-06</b>
System process	2264	33	23.42	+	2.73E-02	167	121	+	<b>1.41E-05</b>
Endocytosis	575	8	5.95	+	2.47E-01	56	31	+	<b>2.03E-05</b>
Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3827	39	39.59	-	5.01E-01	257	205	+	<b>4.43E-05</b>
Synaptic transmission	635	10	6.57	+	1.25E-01	59	34	+	<b>4.53E-05</b>
Protein transport	1646	18	17.03	+	4.39E-01	124	88	+	<b>8.73E-05</b>
Intracellular protein transport	1646	18	17.03	+	4.39E-01	124	88	+	<b>8.73E-05</b>
Cellular amino-acid and derivative metabolic process	367	3	3.8	-	4.73E-01	38	20	+	<b>1.29E-04</b>
Amino-acid transport	77	1	0.8	+	5.50E-01	13	4	+	<b>3.47E-04</b>
Carbohydrate metabolic process	952	17	9.85	+	2.10E-02	76	51	+	<b>4.44E-04</b>
Cell-cell signaling	1374	22	14.22	+	2.82E-02	102	73	+	<b>6.19E-04</b>
Developmental process	3052	40	31.58	+	6.60E-02	202	163	+	<b>7.65E-04</b>
Cellular component organization	1492	18	15.44	+	2.84E-01	108	80	+	<b>1.00E-03</b>
Response to stress	500	9	5.17	+	7.74E-02	44	27	+	<b>1.18E-03</b>
Mitosis	635	7	6.57	+	4.86E-01	52	34	+	<b>2.04E-03</b>
Vitamin transport	95	2	0.98	+	2.58E-01	13	5	+	<b>2.25E-03</b>
Ectoderm development	1469	24	15.2	+	1.81E-02	104	79	+	<b>2.46E-03</b>
Lipid metabolic process	1119	14	11.58	+	2.70E-01	82	60	+	<b>2.92E-03</b>
Cell cycle	1882	20	19.47	+	4.84E-01	128	101	+	<b>3.23E-03</b>
Cellular component morphogenesis	1170	15	12.1	+	2.32E-01	84	63	+	<b>4.42E-03</b>
Anatomical structure morphogenesis	1170	15	12.1	+	2.32E-01	84	63	+	<b>4.42E-03</b>
Visual perception	412	5	4.26	+	4.23E-01	35	22	+	<b>6.00E-03</b>
Carbohydrate transport	187	6	1.93	+	1.39E-02	19	10	+	<b>6.94E-03</b>
Cell surface receptor linked signal transduction	2235	33	23.12	+	2.32E-02	146	120	+	<b>6.98E-03</b>
Response to stimulus	1798	34	18.6	+	<b>4.49E-04</b>	113	96	+	4.29E-02
Immune system process	2630	43	27.21	+	<b>1.50E-03</b>	156	141	+	9.12E-02
Immune response	756	16	7.82	+	<b>5.73E-03</b>	40	40	-	5.14E-01
Macrophage activation	305	9	3.16	+	<b>4.86E-03</b>	16	16	-	5.35E-01
<i>Molecular function</i>									
Binding	6794	76	70.29	+	2.21E-01	467	363	+	<b>3.01E-11</b>
Protein binding	3200	36	33.11	+	3.19E-01	247	171	+	<b>1.10E-09</b>
Catalytic activity	5336	65	55.21	+	7.36E-02	363	285	+	<b>9.65E-08</b>
Transporter activity	942	11	9.75	+	3.84E-01	87	50	+	<b>9.21E-07</b>
Enzyme regulator activity	1187	9	12.28	-	2.11E-01	103	63	+	<b>1.46E-06</b>
Transmembrane transporter activity	897	11	9.28	+	3.26E-01	81	48	+	<b>5.02E-06</b>
Transferase activity	1593	16	16.48	-	5.16E-01	121	85	+	<b>7.65E-05</b>
Kinase regulator activity	320	0	3.31	-	3.55E-02	35	17	+	<b>8.46E-05</b>
Translation factor activity, nucleic acid binding	107	0	1.11	-	3.30E-01	17	6	+	<b>9.55E-05</b>

**Table 2** Continued

	NCBI (19911) <sup>a</sup>	STR (206) <sup>b</sup>	EXP	+/-	P-value	DEP (1065) <sup>c</sup>	EXP	+/-	P-value
Translation initiation factor activity	80	0	0.83	-	4.36E-01	14	4	+	<b>1.46E-04</b>
Nucleic acid binding	3863	43	39.97	+	3.23E-01	253	207	+	<b>2.58E-04</b>
Small GTPase regulator activity	495	4	5.12	-	4.17E-01	45	26	+	<b>5.46E-04</b>
RNA binding	530	3	5.48	-	2.00E-01	47	28	+	<b>6.97E-04</b>
Translation regulator activity	105	0	1.09	-	3.36E-01	15	6	+	<b>7.08E-04</b>
Hydrolase activity	2236	34	23.13	+	1.43E-02	154	120	+	<b>7.34E-04</b>
Guanyl-nucleotide exchange factor activity	160	0	1.66	-	1.90E-01	19	9	+	<b>1.33E-03</b>
Lipid transporter activity	92	0	0.95	-	3.85E-01	13	5	+	<b>1.71E-03</b>
Receptor binding	1235	17	12.78	+	1.42E-01	89	66	+	<b>3.11E-03</b>
RNA splicing factor activity, transesterification mechanism	268	1	2.77	-	2.34E-01	26	14	+	<b>3.31E-03</b>
Helicase activity	159	1	1.65	-	5.10E-01	17	9	+	<b>6.43E-03</b>
Kinase inhibitor activity	122	0	1.26	-	2.82E-01	14	7	+	<b>7.15E-03</b>
Amino-acid transmembrane transporter activity	98	0	1.01	-	3.62E-01	12	5	+	<b>7.60E-03</b>
Deaminase activity	43	0	0.44	-	6.41E-01	7	2	+	<b>9.28E-03</b>
Acyltransferase activity	193	0	2	-	1.34E-01	19	10	+	<b>9.46E-03</b>
Extracellular matrix structural constituent	147	7	1.52	+	<b>9.41E-04</b>	15	8	+	1.47E-02

Abbreviations: DEP, depression genes; EXP, expected (rounded to nearest whole number); NCBI, National Center for Biotechnology Information; STR, chronic stress response genes.

<sup>a</sup>Total number of genes referenced from NCBI.

<sup>b</sup>Stress response genes ( $P < 0.01$ ) per GO term out of 194 total.

<sup>c</sup>Depression genes ( $P < 0.01$ ) per GO term out of 998 total.  
 $P < 0.01$  values are bolded.

protein and transthyretin in the amygdala and/or hippocampus.<sup>39,73</sup> In addition, stress has previously been found to increase the expression of amyloid beta precursor protein<sup>74,75</sup> and neuropeptide Y.<sup>76-78</sup> Our findings, however, also implicate a number of novel 'stress genes'. Among these, the decreased mRNA levels of tumor suppressor protein 53, insulin-like growth factor 2 and hemoglobin beta and alpha a1 are of particular interest. Tumor suppressor protein 53 has been linked to the pathology of Huntington disease,<sup>79</sup> a finding that is connected to the overrepresentation of our chronic stress response genes involved in the Huntington pathway. Insulin-like growth factor 2 is an imprinted gene, described as having both neuroprotective and neurodegenerative properties,<sup>80</sup> as well as involvement in metabolic disorders. Recently, hemoglobin beta and alpha transcripts and proteins have been found in cortical and hippocampal astrocytes, mature oligodendrocytes and a subpopulation of dopaminergic neurons.<sup>81</sup> If the decreased expression of hemoglobin beta and alpha a1 is a manifestation of chronic stress-induced decreases in oxygen storage, these findings would implicate chronic stress in neurodegenerative processes. If, however, they are related to the neurotoxicity of hemoglobins,<sup>82</sup> the decreased expression of hemoglobin beta and alpha a1 and of tumor suppressor protein 53 would propose the heretical idea of chronic stress being neuroprotective.

If depression in general is a result of chronic stress and stress-related factors, these genes should be

shared with the endogenous 'depression transcriptome', as defined by WMI-WLI differences. However, a surprisingly small number of genes were shared between WMI-WLI and NS-CRS differentially expressed genes in the amygdala and hippocampus. Genes showing parallel directional expression changes in the hippocampus between the 'endogenous depression' and the 'chronic stress' gene sets include: *Col1a1*, *Mmp14* and *RGD1305680* (homologous to the *KIAA0240* gene). *Mmp14* (also known as *MT1-MMP*) degrades endogenous beta amyloids<sup>83</sup> and is implicated in the pathogenesis of central nervous system inflammatory disorders. It is also a tethered membrane collagenase, which, together with the involvement of *Col1a1*, implies the significance of extracellular matrix remodeling in depressive behavior and chronic stress. Ontological analysis further and more broadly confirmed the dissimilarity between the endogenous depressive behavior and generic stress response expression profiles. Functional annotation of the genes involved in both states revealed few common biological processes and no common molecular functions between the two gene sets.

This study using animal models contends that endogenous depression and chronic stress response are regulated by independent molecular pathways. In humans, an inference of this finding would be that stress-induced and endogenous subtypes of depression are etiologically distinct. There are both human and animal studies supporting this assumption.

For example, only a subset of depressive phenotypes can be causally attributed to stressful life events, and these phenotypes indicate only a moderately increased risk of developing depression.<sup>84,85</sup> In addition, although the chronic mild stress model is thought of as a model of depression, some animals are resistant to the anhedonic effect of stress.<sup>73,86–88</sup> Similarly, some animals are resilient to learned helplessness in the congenital learned helplessness model of depression,<sup>25,89</sup> suggesting that differences in vulnerability to stress may determine the development of depressive phenotype in these models. Thus, it is possible that stress-susceptible individuals could develop a subtype of depression distinct from endogenous depression; this subtype may show greater comorbidity with other stress-related disorders. The existence of different subtypes of depression is in further agreement with the findings of Krishnan and Nestler,<sup>84</sup> which state that there is no ‘unified theory’ of depression. Should our findings translate to MDD, they suggest that different treatment strategies, dependent on the patient’s depressive subtype, could be beneficial. Furthermore, as most animal models of depression are based on stress, the above findings predict that using various genetic animal models of depression could lead to novel drug targets that may achieve successful treatment of patients in which current treatment methods fail.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgments

This work was supported the Davee Foundation, the RD Foundation, and NIH grant MH077234. The authors also wish to thank Laura Sittig, Elif Tunc-Ozcan, and Timothy Ullmann for helpful suggestions on the manuscript and Drs. Frasier Aird and Claire Will for early work on the endogenous depression model.

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