LETTERS

Glyoxalase 1 and glutathione reductase 1 regulate anxiety in mice

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Anxiety and fear are normal emotional responses to threatening situations. In human anxiety disorders—such as panic disorder, obsessive–compulsive disorder, post-traumatic stress disorder, social phobia, specific phobias and generalized anxiety disorder—these responses are exaggerated. The molecular mechanisms involved in the regulation of normal and pathological anxiety are mostly unknown. However, the availability of different inbred strains of mice offers an excellent model system in which to study the genetics of certain behavioural phenotypes[1–3]. Here we report, using a combination of behavioural analysis of six inbred mouse strains with quantitative gene expression profiling of several brain regions, the identification of 17 genes with expression patterns that correlate with anxiety-like behavioural phenotypes. To determine if two of the genes, glyoxalase 1 and glutathione reductase 1, have a causal role in the genesis of anxiety, we performed genetic manipulation using lentivirus-mediated gene transfer. Local overexpression of these genes in the mouse brain resulted in increased anxiety-like behaviour, while local inhibition of glyoxalase 1 expression by RNA interference decreased the anxiety-like behaviour. Both of these genes are involved in oxidative stress metabolism, linking this pathway with anxiety-related behaviour.

Different inbred mouse strains have different physical and behavioural phenotypes that are heritable and stable[1–3]. We combined gene expression profiling and behavioural testing of multiple highly characterized strains in search of candidate genes for anxiety-like behaviour. We identified several strong candidates and performed follow-up functional studies to demonstrate directly that altered expression levels of the identified genes affected anxiety-like behaviour in mice (Supplementary Fig. 1).

Several methods to test levels of anxiety-like behaviour in mice have been developed and pharmacologically 'validated'; that is, shown to be specifically responsive to agents with proven anxiolytic or anxiogenic effects[4]. We used two such tests to measure anxiety-like behaviour in six inbred mouse strains—the light–dark box test and the open-field test (described in the Supplementary Methods). Strain characterization with both tests was consistent (Pearson coefficient of correlation between the 'open-field time spent in the middle of the chamber' and the 'light–dark box time spent in the light compartment' was high, $r = 0.84$), and showed that A/J, DBA/2J and 129S6/SvEvTac were the most anxious strains and FVB/NJ the least anxious strain (Fig. 1a), as reported previously[5,6]. The behaviour of C3H/HeJ and C57BL6/J animals was intermediate (Fig. 1a). In contrast, although not completely ruling out an association between locomotor activity and anxiety-like behaviour, the strain order for locomotor activity, estimated as the distance travelled in the dark compartment of the light–dark box, was different from the strain order for anxiety-like behaviour (Supplementary Information).

Several methods have been used to show that the amygdala, septohippocampal system, medial hypothalamus, central periaqueductal grey, and frontal and cingulate cortices are important brain structures involved in the regulation of anxiety and fear[7–10]. Based on this information, we selected seven brain regions (the amygdala, bed nucleus of the stria terminalis, cingulate cortex, hippocampus, hypothalamus, periaqueductal grey and pituitary gland) thought to regulate aspects of anxiety-related behaviour, and used oligonucleotide arrays (Affymetrix U74Av2) to assess the expression levels of ~10,000 genes in those regions. To ensure that our experimental methodology and data analysis methods minimized the number of false positives and maximized the reliability of the results, we carefully compared at least two independent replicate samples for each brain region from each strain[11]. Reproducibility between replicates was high (Supplementary Table 1), and the estimated false positive rate was low (0.013%; see the Supplementary Methods for details).

We identified oligonucleotide probe sets that showed statistically significant differences in expression levels between two of the most anxious (A/J and DBA/2J) and the two least anxious (FVB/NJ and C57BL/6J) mouse strains in at least one brain region (see the Supplementary Methods for details). We identified eight probe sets in the hippocampus, 12 in hypothalamus, 33 in pituitary, seven in bed nucleus of the stria terminalis, 19 in periaqueductal grey, 12 in amygdala and 12 in cingulate cortex. These probe sets cover genes that are differentially expressed between the phenotypic extremes, but may not necessarily correlate with anxiety-like phenotypes across all six inbred strains. Therefore, we performed a correlation analysis to identify a subset of genes with expression levels that correlate with anxiety-related phenotypes across all strains (see the Supplementary Methods for details). Nineteen probe sets were identified (Table 1, Fig. 1b and Supplementary Table 2), corresponding to 17 candidate genes (probe sets 93268_at and 93269_at both represented glyoxalase 1 (*Glo1*), and probe sets 96215_f_at and 98525_f_at both represented erythroid differentiation regulator 1 (*Erdr1*)). In addition to the correlation analysis described above, we analysed the data with a standard implementation of a linear mixed-effects model to assess the correlation between expression and anxiety-related behaviour (Table 1 and Supplementary Table 2). Only growth hormone (probe set 92783_at) did not show a statistically significant association using this method. Some of the identified genes showed differential expression across several brain regions, while the majority of the genes were differentially expressed between strains in only a single brain region (Table 1). To independently confirm the differences, we performed quantitative polymerase chain reaction with reverse transcription (quantitative RT–PCR; qPCR) for 11 of the 17

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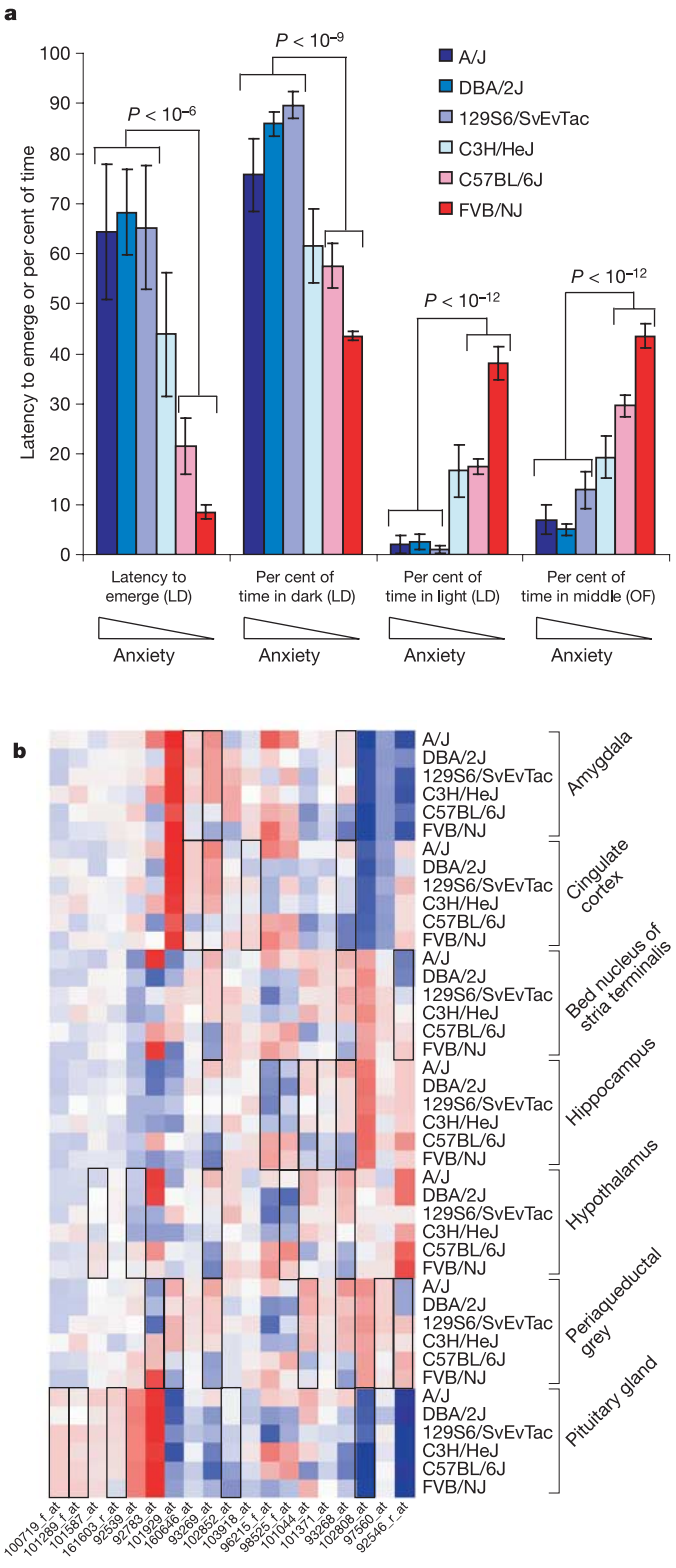
candidate genes (Supplementary Fig. 2). For most of the genes, the differences in gene expression observed by microarray analysis were confirmed by qPCR. Two genes—cadherin 2 (*Cdh2*) and epoxide hydrolase 1 (*Ephx1*)—did not show clear differential expression between the strains by qPCR. It is possible that not all of these differentially expressed genes are involved with the regulation of anxiety. For example, some of them might correlate with the phenotype by chance, so we addressed this question using functional and genetic studies.



Notably, five of the 17 candidate genes were enzymes. Enzyme activity assays were available for three of them. We measured the activities of delta-aminolevulinate dehydratase (Alad), glyoxalase 1 (Glo1) and glutathione reductase 1 (Gsr) from brain homogenates containing combined tissue of hippocampus, striatum and cortex (Supplementary Fig. 2). It seemed that *Alad* mRNA levels in FVB/NJ animals were overestimated by the microarrays, as *Alad* expression and Alad activity did not correlate with anxiety-like behaviour across the strains. In contrast, both Glo1 and Gsr enzyme activities matched the pattern found in both the microarray and qPCR analyses, with highest activities in the most anxious and lowest activities in the least anxious strains. This was particularly intriguing given that reduced glutathione (GSH), the levels of which are maintained by Gsr, is a major antioxidant in the brain. Glo1 uses GSH as a cofactor to detoxify cytotoxic methylglyoxal. Furthermore, erythrocytes from patients with anxiety disorders (such as panic disorder or obsessive–compulsive disorder) may have higher levels of antioxidant enzymes (glutathione peroxidase and superoxide dismutase)[12,13], suggesting that free radicals may have a role in the pathogenesis of anxiety disorders. Oxidative stress has also been implicated in the pathogenesis of other neuropsychiatric diseases, including schizophrenia and major depressive disorder[14,15], and Glo1 is linked to diabetes[16], Alzheimer's disease[17], autism[18] and the regulation of theta oscillations during sleep[19]. A recent study suggested Glo1 might be a biological marker for trait anxiety in bidirectionally crossed mouse lines[20]. Therefore, we sought to determine the role of these candidate genes in influencing anxiety-related behaviour in a complex genetic background.

We analysed the offspring of two different F_1 crosses of the non-anxious C57BL/6J strain and an anxious A/J strain ($AB6F_1$ and $B6AF_1$), in addition to BALB/cByJ inbred mice as this strain was shown to be very anxious. In both open-field and light–dark box tests, F_1 animals derived from the A/J and C57BL/6J crosses showed intermediate levels of anxiety-like behaviour compared to the parental strains (Fig. 2a). We hypothesized that if Glo1 and Gsr exert a strong influence on the phenotype, the activity levels of the enzymes should correlate with the anxiety-related phenotype. As expected, there was a statistically significant correlation between the open-field behaviour and the Glo1 ($P = 0.0005$) and Gsr ($P = 0.009$) enzyme activities, as measured by regression analysis over A/J, C57BL/6J, their F_1 offspring and BALB/cByJ mice (Fig. 2b and c), suggesting that these two enzymes are very strong candidates for regulating anxiety-related behaviours.

To further investigate the role of *Glo1* and *Gsr* in anxiety, we prepared lentiviral vectors to overexpress these genes *in vivo* (Supplementary Fig. 3a). The lentiviral approach was favoured over other viral vectors because lentiviral vectors efficiently transduce central nervous system cells and are not cytotoxic[21,22]. One microlitre of either *Glo1*- or *Gsr*-containing virus, or a green fluorescent protein (GFP)-containing control virus, was injected bilaterally in the region

Figure 1 | Inbred mouse strains have different levels of anxiety-related behaviours. a, Behavioural tests on inbred strains of mice. Test parameters are shown on the *x* axis. The *y* axis shows either the latency to emerge from the dark side to the light side of the light–dark (LD) chamber (zero corresponds to 0 min and 100 corresponds to 5 min), the per cent of time in the dark or light side of the light–dark chamber, or the per cent of time in the middle of the open-field (OF) chamber. See the Supplementary Methods for the test measures and analysis. Values are mean ± s.e.m. *P* values calculated using a two-tailed Student's *t*-test. **b**, A heat map based on the cluster analysis of the 19 probe sets with signals that correlated with the anxiety-related phenotype, and that were significantly different between the most and the least anxious strains (bordered by a black box). The *x* axis shows the probe set identifiers. Mouse strains are organized by tissue and level of anxiety-like behaviour on the *y* axis. Red represents high and blue represents low signal intensity, with a more intense colour showing relatively higher signal intensity.

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Table 1 | Correlation of gene expression patterns with anxiety-related phenotypes in six inbred mouse strains

Probe set	Gene title	Gene symbol	Tissue	Average fold change*	Correlation coefficient (OF behaviour)†	Association *P* value (OF–gene expression)‡
102852_at	Cadherin 2	*Cdh2*	pi	−1.72	0.95	7.7×10^{-4}
161603_r_at	Erythrocyte protein band 4.1-like 4a	*Epb4.1l4a*	pi	−3.98	0.89	2.5×10^{-2}
93268_at	Glyoxalase 1	*Glo1*	am, ci, bn, hi, **hy**, pa	−2.32	0.97	2.6×10^{-5}
93269_at	Glyoxalase 1	*Glo1*	**am**, ci, bn, hi, hy, pa	−2.53	0.94	7.8×10^{-5}
101044_at	Delta-aminolevulinate dehydratase	*Alad*	hi, **pa**	−2.17	0.84	6.0×10^{-5}
160646_at	Glutathione reductase 1	*Gsr*	am, **ci**	−2.83	0.85	2.6×10^{-3}
101371_at	Cleavage and poly-adenylation specific factor 4	*Cpsf4*	hi	−1.90	0.80	5.2×10^{-4}
97560_at	Prosaposin	*Psap*	pa	−1.73	0.80	2.4×10^{-4}
102808_at	Voltage-gated sodium channel type Iβ	*Scn1b*	pi	−2.02	0.77	1.5×10^{-3}
101929_at	Dynein light chain 2	*Dlc2*	pa	−1.85	0.76	3.2×10^{-2}
92539_at	S100 calcium binding protein A10	*S100a10*	hy	1.80	−0.76	2.0×10^{-3}
101289_f_at	Kallikrein 21	*Klk21*	pi	6.74	−0.77	3.0×10^{-2}
101587_at	Epoxide hydrolase 1	*Ephx1*	hy	2.74	−0.78	5.6×10^{-3}
92783_at	Growth hormone	*Gh*	pa	5.20	−0.80	2.9×10^{-1}
103918_at	Solute carrier family 15 member 2	*Slc15a2*	ci	4.27	−0.80	2.6×10^{-6}
92546_r_at	Prostaglandin D2 synthase	*Ptgds*	**bn**, pa	2.67	−0.82	3.1×10^{-4}
100719_f_at	Kallikrein 16	*Klk16*	pi	5.54	−0.83	1.3×10^{-3}
98525_f_at	Erythroid differentiation regulator 1	*Erdr1*	hi, **hy**	2.74	−0.87	3.5×10^{-2}
96215_f_at	cDNA clone MGC:67258	*Erdr1*	hi	3.75	−0.98	3.1×10^{-3}

*Average fold change for the C57BL/6J and FVB/NJ versus A/J and DBA/2J comparisons. Value shown is the average over all tissues showing differential expression.
†In the case of multiple tissues, the most significant value is shown (for the tissue in bold).
‡Based on the linear mixed-effects model analysis. am, amygdala; bn, bed nucleus of the stria terminalis; ci, cingulate cortex; hi, hippocampus; hy, hypothalamus; pa, periaqueductal grey; pi, pituitary; OF, open-field test.

of the cingulate cortex of C57BL/6J and 129S6/SvEvTac mice to overexpress the corresponding genes *in vivo*. These strains were selected because they are widely used in neurobiological research, with C57BL/6J representing a non-anxious strain and 129S6/SvEvTac representing an anxious strain. Injected animals were tested in the open-field test (Fig. 2d–e and data not shown). After testing, mice were allowed to recover for a week, killed, and their brains removed for immunohistochemical and *in situ* hybridization analysis. We confirmed transgene expression associated with stereotaxic injection by *in situ* hybridization (Supplementary Fig. 3b–c).

Overexpression of *Glo1* in the cingulate cortex of the anxious 129S6/SvEvTac strain further enhanced the anxiety-related phenotype. The *Glo1*-expressing mice spent 12% more time near the walls in the open-field chamber compared to the GFP-expressing controls ($P = 0.016$; Fig. 2d). This effect was evident as early as five weeks after injection. Similarly, 129S6/SvEvTac mice overexpressing *Gsr* in the cingulate cortex were more anxious than GFP-expressing controls, although the effect was on the border of statistical significance ($P = 0.054$; Fig. 2d). The less-anxious C57BL/6J mice injected with the *Gsr* lentivirus also showed an increase in anxious behaviour, spending 16% more time near the walls in the open-field chamber compared to GFP-expressing controls ($P = 0.003$; Fig. 2e). However, overexpression of *Glo1* in the C57BL/6J background did not increase the anxiety-related behaviour compared to GFP controls ($P = 0.212$; Fig. 2e). The behaviours of the three groups (*Glo1*-, *Gsr*- and GFP-expressing animals) were significantly different at five weeks after injection in 129S6/SvEvTac mice ($P = 0.047$), and at seven weeks after injection in C57BL/6J mice ($P = 0.040$), as shown by a Kruskal–Wallis non-parametric analysis of variance (ANOVA).

To further prove that the expression level of these genes modulates anxious behaviour, we tested whether inhibition of *Glo1* gene expression led to a decrease in anxiety-like behaviour using lentiviral vectors that expressed an siRNA (small interfering RNA) against *Glo1* (siGlo1). A control vector was used that expressed an siRNA against the human *p53* gene (sihp53)[23], which has been shown not to affect the expression of mouse *p53* (Supplementary Fig. 4; O.S. and I.M.V., unpublished results). The 129S6/SvEvTac and C57BL/6J strains of mice were injected with either a virus expressing siGlo1 or sihp53. Five weeks later, animals were tested using the open-field test. The 129S6/SvEvTac mice injected with siGlo1 virus spent 49% more time in the middle of the chamber compared with control animals injected with the sihp53 virus ($P = 0.036$; Fig. 2f). Likewise, C57BL/6J mice injected with siGlo1 virus spent 38% more time in the middle of the chamber compared with control animals injected with the sihp53 virus ($P = 0.0002$; Fig. 2f), indicating that inhibition of *Glo1* expression in the cingulate cortex reduces levels of anxiety-like behaviour. We confirmed transgene expression associated with stereotaxic injection by visualizing GFP expression associated with lentiviral infection (Supplementary Fig. 3d).

The results of our lentivirus experiments show that overexpression of either *Glo1* or *Gsr* in the cingulate cortex increases, while inhibition of *Glo1* expression by siRNA decreases, the level of anxiety-like behaviour of mice. These results strongly support the hypothesis that changes in the expression levels of *Glo1* and *Gsr* in the brain lead to a significant effect on anxiety-related behaviour, and establish a causal role for these genes, which are both part of a pathway that regulates oxidative stress, in the genesis of anxiety-like behaviour.

We have shown that gene expression profiles of specific brain regions of anxious and non-anxious mice differ significantly. Our expression-based approach is expected to complement traditional QTL (quantitative trait loci) mapping: genes with expression levels that are correlated with the trait of interest and physically reside in close proximity to a QTL for the trait are good candidates for genes directly responsible for the QTL[24,25]. In fact, several of our candidate genes reside within chromosomal regions with identified QTLs for anxiety-related behaviour[26,27] (Supplementary Table 2). The newly identified genes should further our understanding of the specific genes, pathways and mechanisms that are important for the regulation of normal and pathological anxiety in mice and humans.

METHODS

Animals. Seven-week-old male mice were obtained from the Jackson Laboratory (A/J, BALB/cByJ, C3H/HeJ, C57BL/6J, DBA/2J, FVB/NJ and B6AF1/J) or from Taconic Farms (129S6/SvEvTac). $AB6F_1$ animals were bred at the Salk Institute using parental animals derived from the Jackson Laboratory. Animals were singly housed for one week before behavioural testing or dissections. All animal procedures were approved by the Salk Institute for Biological Studies institutional animal care and use committee. Different animals were used for behavioural testing and gene expression profiling in order to measure baseline gene expression differences.

Behavioural testing. Anxiety-related behaviour was measured using the light–dark box test and the open-field test (see the Supplementary Methods for details).

Tissue collection and RNA preparation. Animals were killed by cervical

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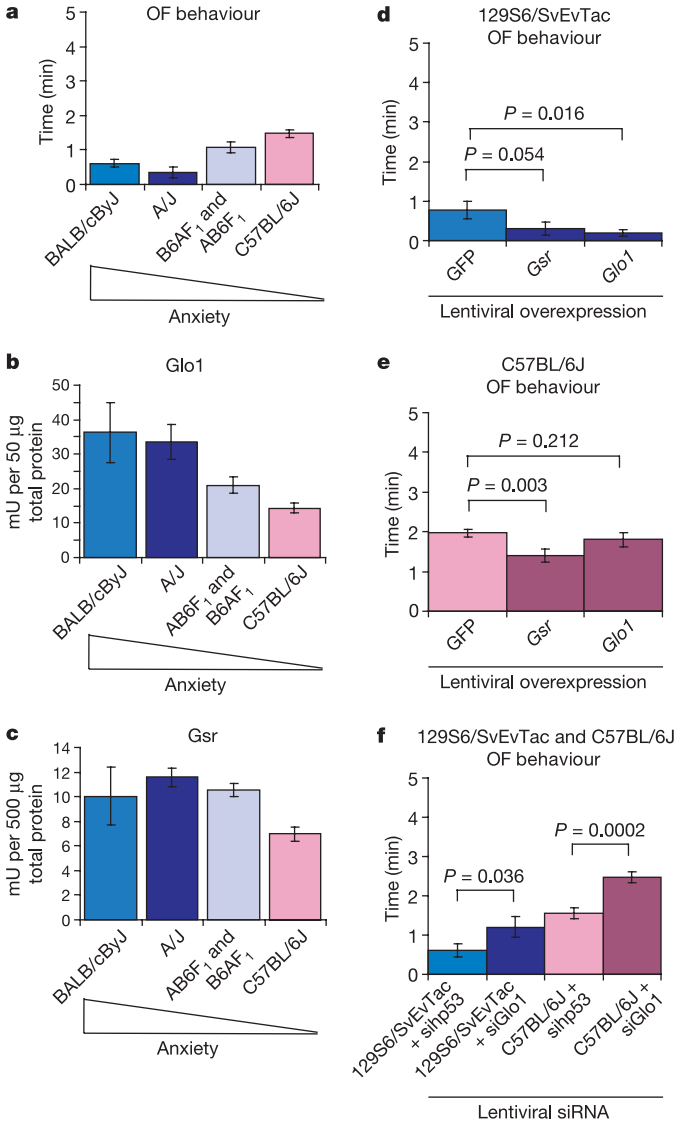

Figure 2 | Glyoxalase 1 (Glo1) and glutathione reductase 1 (Gsr) regulate anxiety-like behaviour in inbred mouse strains. a, Open-field (OF) behaviour. Mouse strains are shown on the *x* axis. Time spent in the middle of the open-field chamber is shown on the *y* axis. Values are mean ± s.e.m. **b**, Glo1 and **c**, Gsr brain enzyme activity (mean of two to four animals ± s.d.). See the Supplementary Methods for a description of the units. **d–f**, Open-field behaviour of *Glo1*-, *Gsr*- or GFP-overexpressing 129S6/SvEvTac mice five weeks after injection of the lentivirus (**d**); *Glo1*-, *Gsr*- or GFP-overexpressing C57BL/6J mice seven weeks after injection (**e**); and siGlo- or sihp53-expressing 129S6/SvEvTac and C57BL/6J mice five weeks after injection (**f**). In each case, the *x* axis shows the name of the injected lentivirus. Time spent in the middle of the open-field chamber is shown on the *y* axis. Values are mean ± s.e.m. *P* values calculated using a one-tailed Student's *t*-test.

dislocation. All dissections were performed between 11.00–17.00 h on a Petri dish filled with ice using a dissection microscope. The dissected brain regions for gene expression analysis included the amygdala, cingulate cortex, hypothalamus, hippocampus, pituitary, periaqueductal grey and bed nucleus of the stria terminalis. Hippocampus samples were directly frozen on dry ice and stored at −80 °C. The smaller brain structures were collected in RNA Later buffer (Ambion), and samples from 2–5 animals were pooled and stored at −80 °C. The extraction of total RNA from the tissues was performed using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Only samples with an absorbance ratio at 260 nm/280 nm (A_{260}/A_{280}) greater than 2.0 in TE buffer were used for further experiments.

Microarray experiments. Gene expression levels were measured using the Murine Genome U74Av2 arrays (Affymetrix). Bed nucleus of the stria terminalis, hippocampus, hypothalamus, periaqueductal grey and pituitary gland samples were labelled using10 µg of total RNA as the starting material. Owing to the small size of amygdala and cingulate cortex, samples from these tissues were labelled using 50 ng of total RNA as the starting material, using two rounds of complementary DNA synthesis and *in vitro* transcription (IVT). Labelling of samples, hybridization and scanning were performed as described[28]. Two-round labelling was performed using the MessageAmp kit (Ambion) according to the manufacturer's instructions, with the exception that the second IVT was done using the Enzo BioArray high yield RNA transcript labelling kit (Enzo Life Sciences).

Data analysis. See the Supplementary Methods for further details concerning the analysis of differentially expressed genes and the determination of reproducibility between measurements, as well as the regression analysis between the behavioural results and enzyme activity levels.

Quantitative RT–PCR. PCR reactions were done using the SYBR Green master mix (Applied Biosystems) in an ABI Prism SDS 7900 HT machine (Applied Biosystems) as described in the Supplementary Methods.

Enzyme activity assays. Eight-week-old mice were killed by decapitation and their cortex, hippocampus and striatum dissected under a dissection microscope, frozen on dry ice, and stored at −80 °C. The enzyme activity levels of Alad, Glo1 and Gsr were determined as described in the Supplementary Methods.

Lentivirus-mediated gene transfer. Plasmids were constructed for the production of lentiviral vectors that expressed either *Glo1* or *Gsr* with a carboxy-terminal HA-tag, or GFP, in the overexpression experiment. We sequenced the cDNA of *Glo1* and *Gsr* in order to find single nucleotide polymorphisms between the strains (see the Supplementary Methods and Supplementary Information). For the overexpression experiment, a variant of *Glo1* from the A/J strain was cloned. For the siRNA experiment, lentiviral vectors were constructed that expressed siRNA against *Glo1* (siGlo1) or human *p53* (sihp53) from the human H1-RNA promoter as described before (O.S. and I.M.V., unpublished results and ref. 23) (Supplementary Fig. 3a). Further details about virus production are given in the Supplementary Methods. A total of 50 129S6/SvEvTac and 50 C57BL/6J male mice were obtained from Taconic Farms or the Jackson Laboratory, respectively, at five weeks of age, and housed five mice per cage. After one week of acclimatization, mice were injected bilaterally with 1 µl (1.1×10^6 transducing units) of either HA–*Glo1*, HA–*Gsr*, GFP, siGlo1 or sihp53 virus (ten animals of both strains per construct) into the cingulate cortex using a stereotaxic frame. The stereotaxic coordinates were: 1.4 mm rostral to bregma, 0.5 mm lateral to midline, and 1.5 mm ventral from the dural surface. Four weeks after injection, mice were separated into individual cages. A few animals died after the injections, and the final number of animals used for further experiments are detailed in the Supplementary Methods. The open-field behavioural test was conducted five weeks and seven weeks after injection in the case of the overexpression experiment, and five weeks after injection in the case of the siRNA experiment. Mice were allowed to recover for a week, after which time they were killed and their brains were collected for the immunohistochemical or *in situ* hybridization analysis (see the Supplementary Methods for details).

Software tools. Further details on the TeraGenomics microarray analysis tool are available at http://www.teragenomics.com. The Bullfrog software can be downloaded from http://www.barlow-lockhartbrainmapnimhgrant.org/.

Received 18 April; accepted 23 September 2005.
Published online 23 October 2005.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank Information Management Consultants and Teradata (NCR) for donating and programming of the TeraGenomics database; Dan Lockhart, M. Zapala and N. Schork for software development and statistical analysis; B. Stoveken for brain dissections; J. Airas for plasmid cloning; N. Tonnu for lentivirus production; F. Bloom, J. Reilly, W. Young, W. Vale and T. Carter for their insight; and members of the Barlow laboratory for discussions and technical assistance. This work was supported by grants from NIMH (to C.B. and D.J.L.), NINDS (to C.B.) and the Academy of Finland (to I.H.).

Author Contributions D.J.L. and C.B. conceived of and initiated the project. I.H., D.J.L. and C.B. designed the research. I.H. and R.S.T. performed the microarray, enzyme activity, sequencing and real-time qPCR experiments. I.H. and R.H. performed the behavioural analyses and lentivirus injections. I.H., J.M.R., J.A.E. and C.B. designed and J.M.R. performed the *in situ* hybridization experiments. I.H., R.A.M., O.S., I.M.V. and C.B. designed the lentivirus experiment, and R.A.M., O.S. and I.M.V. contributed the lentivirus vectors. I.H., E.E.S., C.B. and D.J.L. analysed the data. I.H., E.E.S., D.J.L. and C.B. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Author Information Microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through the GEO series accession number GSE3327. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to C.B. (cbarlow@braincellsinc.com).

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