MOLECULAR CHARACTERIZATION OF THE STRESS NETWORK IN THE HUMAN BRAIN

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ABSTRACT

The biological mechanisms underlying inter-individual differences in human stress reactivity remain poorly understood. We aimed to identify the molecular underpinning of neural stress sensitivity. Linking mRNA expression data from the Allen Human Brain Atlas to task-based fMRI revealed 261 differentially expressed genes in brain regions differentially activated by stress in individuals with low or high stress sensitivity. These genes are associated with stress-related psychiatric disorders (e.g. schizophrenia and anxiety) and include markers for specific neuronal populations (e.g. VIP, CCK, and NPY), neurotransmitter receptors (e.g. HTR1A, CHRNA3), and signaling factors that interact with the glucocorticoid receptor and hypothalamic-pituitary-adrenal axis (e.g. CRH, NEUROD2, PACAP). Overall, the identified genes potentially underlie altered stress reactivity in individuals at risk for psychiatric disorders and play a role in mounting an adaptive stress response, making them potentially druggable targets for stress-related diseases.

SIGNIFICANCE STATEMENT

Even though stress increases the risk for almost all psychiatric disorders, large inter-individual differences exist. The molecular mechanisms in humans underlying the heterogeneity in stress sensitivity remain poorly understood. Therefore, this study aimed to disentangle the brain underpinning of stress reactivity in individuals at increased risk for psychiatric disorders. To this end, we used a novel approach by combining neuroimaging data with gene expression data. Overall, we report new molecular pathways that determine how individuals respond to stress and that may be involved in the increased risk for psychiatric disorders. Identifying the molecular mechanisms how the brain can elicit an adaptive stress response is important to understand who is at risk, but also reveal potentially druggable targets for stress-related disorders.

INTRODUCTION

Stress is a major risk factor for the development of a wide range of psychiatric disorders, including schizophrenia and depression.[1] Inter-individual differences in how the brain responds to stress depend on intrinsic (*e.g.* genetic and developmental) as well as on extrinsic (*e.g.* hormonal) factors.[2] The neural correlates underlying stress reactivity are currently a growing topic of investigation.[3-5] In healthy individuals, acute stress causes a shift in neural networks by suppressing the executive

control network and activating the salience network and default mode network (DMN).[6, 7] One hypothesis is that stress vulnerability is the result of maladaptive changes in the dynamic response of these neural networks, either during the acute phase, during the recovery period in the aftermath of stress, or both.[2] Moreover, acute social stress deactivates the DMN in the aftermath of stress during emotion processing in healthy controls but not in siblings of schizophrenia patients who are at-risk for several psychiatric disorders.[8, 9] Yet, the molecular mechanisms underlying differences in brain reactivity to stress in humans remain unknown as access to the tissue of interest in humans is limited.

Nevertheless, stress-related brain regions and networks as identified by fMRI can be further characterized based on transcriptomic signatures. Mapping gene expression atlases of the healthy brain to imaging data allows the identification of the molecular mechanisms underlying imaging phenotypes. Previous studies have identified gene expression patterns associated with structural brain changes in autism spectrum disorders, Huntington's disease and the onset of schizophrenia.[10-13] Similarly, mapping resting-state fMRI and connectivity data onto gene expression atlases has led to identification of molecular profiles underlying these fMRI networks.[14-16]

In this study, we examined the putative molecular signatures of brain regions linked to stress reactivity. We linked gene expression data from the Allen Human Brain Atlas (AHBA) to an fMRI-stress network (Figure 1). In short, we found that the stress network was enriched for genes associated to specific subtypes of neurons (*i.e.* components of the cortical circuitry) with genetic relevance for psychiatric disorders, and for signaling factors and proteins that interact with the activation of the Hypothalamic-Pituitary-Adrenal axis (HPA-axis) and response to glucocorticoids. These all constitute potential targets for directed pharmacotherapy in stress related disorders.

METHODS

Defining the stress network

Based on a previous study, we selected brain regions that were differentially affected by stress in individuals with high and low stress sensitivity.[6] These regions include key nodes of the DMN (posterior cingulated cortex/precuneus and medial prefrontal cortex) and salience network (anterior insula), as well as the superior temporal gyrus, middle temporal gyrus, middle cingulate gyrus, ventrolateral prefrontal cortex, precentral gyrus and cerebellar vermis (Figure 2A).

Allen Human Brain Atlas (AHBA)

Gene expression data from six healthy brains were acquired from the AHBA.[17] In this microarray dataset, probes were mapped to genes as previously described.[18] Z-scores for normalized gene expression levels from the AHBA were calculated separately for each of the six individual brains. Gene expression data were linked to an fMRI-based stress network according to the MNI coordinate system, such that samples of the AHBA exactly overlap with the corresponding fMRI voxels. For all samples in the AHBA, we determined whether they were located in the stress network for all six donors separately. The gene expression levels of the AHBA samples were extracted and resulted in expression data of 19,992 genes in 127 and 3,575 brain samples in- and outside the stress network, respectively.

Differential gene expression in the stress network

To identify genes differentially expressed between the stress network and the rest of the brain, we analyzed each of the six brain donors separately. For each gene, we combined effect sizes (difference in mean expression between the brain stress network and the rest of the brain) across donors using a meta-analysis approach from the 'metafor' 2.0-0 R package. In brief, a random effects model was

used, taking into account the within-brain and between-brain variance, which was estimated with the Dersimonian-Laird model. Variances and confidence intervals needed for the meta-analysis were calculated using the escalc-function. Given the large difference in the transcriptional profile of the cerebellum compared to the rest of the brain[19], we excluded the cerebellum from the analysis. Genes were considered to be differentially expressed at an BH-adjusted p-value < 0.05 (Benjamini-Hochberg (BH) correction) and effect size of $\log_2$(fold-change) > |1|.

Differential expression was determined for the stress network altogether as one mask. In addition, four brain regions in the stress network (frontal gyrus, cingulate gyrus, cerebellum and the hippocampal formation) included sufficient samples (> 2 in the mask) in- and outside the stress network to perform the analysis on these particular structures separately.

Gene Ontology (GO) enrichment analysis

To characterize the functionality of the differentially expressed genes, a GO enrichment analysis was performed. The list of unranked differentially expressed genes was uploaded to GOrilla (Gene Ontology Enrichment Analysis and Visualization Tool).[20] As a background list, the top 20% of genes with the highest expression level in the cerebrum was used, to correct for non-selective ontologies. GO terms were considered significant when the p-values < 0.001 (Fisher's exact test) after BH-correction.

Cell type enrichment analysis

We assessed whether the differentially expressed genes were enriched for cell type markers.[21] Genes with a 20-fold higher expression in neurons (628 marker genes), oligodendrocytes (186 marker genes), astrocytes (332 marker genes), microglia (520 marker genes) and endothelial cells (456 marker genes) were considered to be markers for that cell type. Since most of our AHBA samples

were located inside the cortex, we used a set of brain-region-specific markers and focused on 18 cortical cell types.[22] Details on markers can be found on https://pavlab.msl.ubc.ca/data-and-supplementary-information/supplement-to-mancarci-et-al-neuroexpresso. Finally, to assess which neuronal cell types might be involved in stress sensitivity, single cell RNA sequencing data of the middle temporal gyrus of the human neocortex from the Allen Brain Institute[23] (http://celltypes.brain-map.org/rnaseq/human) were used. The sum of the $\log_{10}$ values of the counts per differentially expressed gene were calculated for each cell cluster separately.

Enrichment analysis of disease-associated genes

To assess whether the differentially expressed genes are associated to stress-related psychiatric disorders, a disease-associated gene enrichment analysis was performed based on existing Genome-Wide Association Studies (GWAS) including schizophrenia,[24,25] stress-related diseases such as Post-Traumatic Stress Disorder, as well as non-stress-related diseases (e.g. Huntington and osteoporosis) based on disease gene sets from DisGeNET[26]. As non-disease control conditions, genes associated to height and waste-hip ratio were included in the analyis.[27, 28]

To assess the enrichment of disease-related gene sets in intercellular signaling genes, neuropeptides and receptor genes were selected from the differentially expressed genes. Odds ratios (ORs) were calculated for the set of neuropeptides and receptors for each disease as a measurement of effect size, (i.e. the increased chance of a peptide or receptor being present in the set of differentially expressed genes). For this, the number of receptors found within the trait was compared to all the receptors in the genome, based on the gene annotation of the AHBA. Gene names that included the word 'receptor' were selected and this list was manually verified whether the gene was a receptor or a modulator. The ORs for the neuropeptides were calculated in the same way, based on a list of neuropeptides available from NeuroPep.[29]

Mineralocorticoid and glucocorticoid DNA binding loci

MR and GR DNA binding loci under stress in the rat hippocampus were previously assessed.[30] We identified sets of genes with GR-specific, MR-specific and GR-MR-overlapping DNA binding loci, i.e. potential target genes. To predict glucocorticoid sensitivity of our differentially expressed genes, we assessed whether these sets of targets were enriched among the differentially expressed genes.

Enrichment statistics for GO, cell type, disease-associated genes and receptor binding

Enrichments were assessed based on Fisher's Exact Tests and odds ratios (ORs) were calculated as a measurement of effect size for the enrichments. An OR of 1 indicates no effects, whereas an OR > 1 and 0 < OR < 1 reflects enrichment and depletion, respectively. All p-values were corrected for multiple testing using Benjamini-Hochberg method and a BH corrected p-value < 0.05 was considered to be significant, unless stated otherwise.

Literature search on the differentially expressed genes

For all differentially expressed genes, a literature search was performed to determine whether they were already known to be associated to stress, the HPA-axis or glucocorticoid responsiveness with the PubMed search: ("gene"[all fields]) AND ("stress"[all fields] OR "HPA-axis"[all fields] OR "cortisol"[all fields] OR "glucocorticoid"[all fields] OR "GR"[all fields]). Interactions between genes were found by searching gene pairs in Google Scholar. Based on the information acquired from literature, a putative pathway that indicates how the differentially expressed genes may affect stress reactivity was drawn with yEd Graph Editor (yWorks GmbH, Tübingen, Germany).

RESULTS

Differentially expressed genes in the stress network

We identified the gene expression signatures of the stress network with altered stress-induced activity by determining which genes are differentially expressed in the stress network compared to the rest of the cerebrum. Using a meta-analysis approach to combine results across all donors of the AHBA (n=6), we identified 261 differentially expressed genes (BH-adjusted $p < 0.05$ and $\log_2$(fold-change) > |1|, Figure 2B, left panel; Table S1). Among those genes, 229 were higher expressed, while the other 32 genes were lower expressed in the stress network compared to the rest of the cerebrum.

The differentially expressed genes showed high expression values in the cortex but not the hippocampus, and low expression levels in non-cortical areas (Figure 2C and Figure S2). The two most differentially expressed genes in the stress-specific brain regions are D-amino acid oxidase (*DAO)* (BH-adjusted p-value = $4.99*10^{-17}$, $\log_2$(FC) = -2.40) and Transmembrane protein 155 (*TMEM155)* (BH-adjusted p-value = $4.90*10^{-55}$, $\log_2$(FC) = 3.11)*. TMEM155* is highly expressed in the brain, specifically in the cerebral cortex and claustrum (Figure S3).[31] Its biological function, however, remains unknown, largely because there are no orthologs in the Mus musculus or the Rattus norvegicus. *TMEM155* orthologs are, however, found in chimpanzee[32] and rhesus macaque[33], with similar brain expression patterns between the human and macaque (Figure S3B and S3C).

Functionality and cell-type specificity of differentially expressed genes in the stress network

A GO term enrichment analysis was performed to assess whether the differentially expressed genes in the stress network are enriched for specific functions. The differentially expressed genes were enriched for GO terms involved in neuronal development and neurogenesis, synaptic signal transmission, learning and memory, behavior and glutamate receptor signaling (Figure 3A and Table

S4). Genes involved in most processes based on GO terms (at least assigned to five out of twelve GO terms) include *CRH, MEF2C, NETO1, PLK2, NEUROD2, NR2E1, SERPINF1, CHRNA3, ISLR2, CUX1* and *TBR1*. Enrichment analysis for cellular components indicated that the proteins coded by the differentially expressed genes were mainly found at the synapse, reflecting the high expression of the genes in the synapse-dense cerebral cortex.

Next, we identified the specific cell types underlying the differential gene expression levels in the stress network using enrichment analysis of cortical cell-type markers.[21] Enrichment was found for neuronal cell markers ($p = 0.0414$, BH corrected), including *WIF1, CHRNA3, TAC3, VIP, C1QL1, SLITRK6, NEUROD6, KCTD16, TBR1, CREG2* and *DLX1*. The list of differentially expressed genes included a few astrocytes markers (*AQP9, EMX2, KIRREL2, NR2E1* and *LHX2*) and oligodendrocytes (*EBF3*), but was not significantly enriched ($p = 0.4202$; $p = 0.5267$, BH corrected). Moreover, we found that neuronal markers showed a similar distribution in a t-Distributed Stochastic Neighbor Embedding (t-SNE) map of all genes across the whole brain as the differentially expressed genes, indicating that neuronal markers and the differentially expressed genes show the same expression patterns across the brain (Figure 3B) and thus differential activity may depend on neuronal gene expression.

We further assessed whether the set of differentially expressed genes was particularly expressed in a neuronal subtype. Interestingly, five out of the 22 known thalamic cholinergic neuron markers were found in the set of the differentially expressed genes (*CHRNA3, IRX2, NPPA, TYRP1* and *ZIC4*, BH-adjusted p-value = $3.3*10^{-4}$; Figure 3C). This finding in thalamic cholinergic neuron markers is independent of the number of thalamic samples inside and outside the brain regions vulnerable to stress, showing that this result is not biased by the amount of thalamic samples involved in the maladaptive stress response. Additionally, some of the differentially expressed genes are reported to be part of the molecular fingerprint of particular cell types in the human cortex.[23] For example, *RORB* represents a subclass of excitatory neurons present in the cortex and was modestly enriched in differentially activated brain regions. *VIP* represents one of the four main subclasses of inhibitory

cortical neurons and was quite substantially overrepresented (BH-adjusted p-value = $5.73*10^{-69}$, $\log_2$(FC) = 1.66). Markers for smaller subsets of inhibitory neurons included overrepresentation of *NPY* (a small set of deep projecting inhibitory layer 3 neurons; BH-adjusted p-value = $3.53*10^{-25}$, $\log_2$(FC) = 1.60) and *SERPINF1* (a subset of inhibitory neurons in layer 1; BH-adjusted p-value = $7.83*10^{-37}$, $\log_2$(FC) = 1.27), and underrepresentation of *CBLN1* (a subset of VIP expressing inhibitory neurons in layer 4; BH-adjusted p-value = $1.85*10^{-11}$, $\log_2$(FC) = 1.20). Using a human-specific single cell RNA-sequencing data of the medio-temporal gyrus[23], we found the differentially expressed genes to be mainly enriched in glutamatergic excitatory neurons compared to GABAergic and non-neuronal cells, using a Wilcoxon rank test (p-value = $2.2*10^{-}16$, Figure 3D).

Differentially expressed genes in stress network are associated to stress-related diseases

We hypothesized that the differentially expressed genes in the stress network would be associated to the genetic background of psychiatric disorders, particularly for stress-related brain disorders, as stress plays a major role in the development of these disorders. Using genetic variants from GWAS of the Genomics of Psychiatry Consortium[24, 25], we assessed whether schizophrenia-associated risk loci are enriched in the set of differentially expressed genes. Indeed, schizophrenia risk genes were enriched in the differentially expressed genes in the stress network (Fisher Exact test, BH-adjusted p-value = $2.4*10^{-3}$). The schizophrenia risk genes *KCNV1, DOC2A, NGEF, NRGN, MEF2C, BCL11b, SATB2, FAM5B, CHRM3, CHRM4* and *TAC3* were present in our differentially expressed genes, and all except one (*CRHM4*) were higher expressed in the brain regions vulnerable to stress. Furthermore, gene-disease associations from DisGeNet, a manually curated database, were used to assess risk gene enrichment for psychiatric, brain and non-brain diseases and non-disease traits. Enrichment was found for neuropsychiatric disorders (schizophrenia, schizoaffective disorder, and anxiety disorders) and other brain diseases (epilepsy). However, no gene enrichment was found for non-brain diseases (e.g. osteoporosis) and non-disease traits (e.g. height and waste-hip-ratio; Figure 4). Thus,

differentially expressed genes in the stress network are predominantly involved in genes relevant for stress-related diseases but not in non-brain-related disorders and traits.

Interestingly, the set of 261 differentially expressed genes in the stress network included a considerable number of neuropeptides and receptors. Apart from their use as markers for specific cell types (e.g. NPY, VIP, and CCK[23]), these are important for signaling in the brain (e.g. NPY stimulates the release of CRH in the hypothalamus[34]) and some of them are known to be involved in the regulation of stress.[35, 36] Therefore, we assessed whether there were more neuropeptides and receptors in our set of genes than you would expect by chance. For both the neuropeptides and the receptors, we found higher odds ratios for brain and psychiatric disorders, with the biggest effect sizes in psychiatric disorders (Figure 4). However, none of the effect sizes were significant.

Cortisol sensitivity of the stress network

The enrichment of the neuronal GO terms in our set of genes and the association with stress-related diseases indicates that the differentially expressed genes in the stress network are relevant for stress and may be responsive to the pivotal stress hormone cortisol. To investigate glucocorticoid sensitivity, we compared our list of differentially expressed genes with genes that show a DNA binding site for the glucocorticoid- and/or mineralocorticoid receptors (GR and MR) in the rat hippocampus by Chromatin Immunoprecipitation sequencing after stimulation with the endogenous steroid corticosterone.[30] Differentially expressed genes that showed DNA binding loci for the GR exclusively are: *STON1, HS3ST2, HTR2A, ZNF831, CACNG3, NPTX2, EPHB6, LRRC7, KCNC2, HTR1A, NETO1, CYP26A1, NCALD, EMX2, CXCL14* and *RORB* (16/1450 binding sites, BH-adjusted p- value = $2.97*10^{-2}$). The differentially expressed genes *HSPB3, EGR3, NEUROD2, TBR1, SLC26A6, SLIT1, PCDH20, MAST3, BCL11b, SCN3B, TFAP2B, MAB21L1* and *CHN1* have DNA binding loci for both the GR and the MR (13/475 binding sites, BH-adjusted p-value = $9.8*10^{-3}$). There was no significant MR DNA binding loci enrichment (*IRX2, IRX3, PIRT, CBLN1, KCNV1, KCNS1, ICAM5, FHL2, DLGAP, ST6GALNAC5,*

RFTN1, KCNQ5, KCNH1 and *LMO7*; 14/1918 binding sites, BH-adjusted p-value = 0.46). These results indicate that differentially expressed genes in brain the stress network are enriched for DNA-binding loci of GR but not MR, further consolidating the relevance of these brain regions in aftermath of the responses to acute stress when the GR plays a dominant role.

Putative molecular pathway as the basis for inter-individual differences in stress reactivity

To create a more comprehensive possible pathway for the molecular mechanisms underlying human stress reactivity, we performed a thorough literature search on PubMed for all differentially expressed genes to assess whether previous studies have found these genes to be regulated by or regulating the HPA-axis. We found that 53 of our differentially expressed genes were earlier described to interact with the HPA-axis (Table S5), with a subset of 36 genes reported to interact with each other (Figure 5, Table S5). A putative pathway with factors involved in stress reactivity was build based on these 36 genes (Figure 5).

DISCUSSION

In this study, we identified genes and pathways in the stress network based on an fMRI-based study involving acute stress exposure. By combining fMRI data to gene expression data, we found 261 differentially expressed genes involved in neuronal processes and enriched in stress-related psychiatric disorders. Moreover, the enriched genes included several neuropeptides and neurotransmitter receptors with a predominant GR regulation and substantial links to HPA-axis activity. This gene set uncovered by combining human gene expression and neuroimaging results give important new insights into the putative neural populations and mechanisms underlying stress vulnerability in humans.

Our results point to the involvement of (cortical) cell type markers in differential stress reactivity. For example, we found enrichment for *NPY* which is a unique marker for deep projecting inhibitory layer

3 neurons in the human cortex.[37] Moreover, the differentially expressed genes are in general highly expressed in excitatory glutamatergic compared to inhibitory GABAergic neurons.[23] Thus, glutamate signaling seems to be involved in a more global level, whereas differential activity of specific GABA-ergic neurons underlies differential reactivity to stress. Specific targeting of these GABAergic populations, based on their receptor repertoire, may help to separate primary from secondary changes in the cortical circuitry.

For genes that do not represent specific neuronal subtypes, changed expression levels may reflect differential responsiveness based on more generic signaling pathways. This may, in particular, be the case for the identified stress-related genes with a genetic association to schizophrenia. One of the most differentially lower expressed genes is the *DAO,* which degrades the NMDA receptor co-agonist D-serine. Glutamate and the NMDA-receptor are involved in stress-associated psychiatric disorders, such as post-traumatic stress disorder and schizophrenia.[38] NMDA also mediates the release of CRH in the amygdala, as one of the central modulators of the HPA-axis.[39] The relatively low expression levels of *DOA* in the stress-vulnerable brain regions may thus reflect higher levels of D-serine and associated NMDA receptor activation, in conjunction with disturbed stress responses in at-risk subjects.

Genes with high expression levels in the regions vulnerable to stress include neuropeptides and neurotransmitter receptors, which may be directly targeted to modify the activity of these brain regions. Apart from its potential role as a cell type marker, NPY is thought to mediate stress resilience[40] and contrary, low levels of NPY result in less stress resilience.[41] We found that *NPY* was higher expressed in the stress network than in the rest of the cerebrum. Targeting the NPY system may therefore normalize aberrant responses as observed in genetically vulnerable individuals. Also a number of noradrenergic, serotonergic, and muscarinic cholinergic receptors are differentially represented in the stress network. All these factors may well have a role in regulating neuronal network activity during maladaptive stress responses.[42-44] Of note, the excitatory 5-HT2A receptors

are overrepresented in brain regions that failed to shut off after stress in at-risk subjects. Antagonism of this receptor is common between several antipsychotic and antidepressant drugs, and normalizing the activity of these brain regions after stressor exposure may be part of their therapeutic mechanism. Another striking gene related to differential stress reactivity is *TMEM155*. It is the most overrepresented gene in our results, indicating that it may be involved in the regulation of neurobiological processes underlying stress reactivity, although its function is currently unknown.

The enrichment analysis of gene ontology terms suggests that the list of differentially expressed genes play a role in stress vulnerability and risk for psychiatric disorders. For example, prenatal chronic stress has consequences on nervous system development as shown in mice.[45-47] Moreover, disruption of neuronal plasticity[48] and learning and memory impairments are induced by a prolonged stressor[49] and common symptoms of stress-related psychiatric disorders.[50] Of note, we found very similar results after exclusion of the genes that had already been associated to stress-related disorders (58 genes) from the enrichment analysis. This shows that our results are not fully dependent on the genes already linked to stress.

Furthermore, we found that differentially expressed genes in the stress network are enriched for DNA-binding loci of the GR but not MR. GR is thought to facilitate recovery and adaptation in the aftermath of stress[51] and polymorphisms as well as post-translational modifications alter susceptibility for stress-related psychiatric disorders.[52, 53] Brain regions related to differential stress reactivity were detected around 30 minutes after acute stress exposure when cortisol levels peak and GR action is becoming dominant over MR action. It was previously found that both groups had a similar cortisol response to stress[54], pinpointing the importance of sensitivity of the GR target genes rather than activating hormone levels. The link with GR but not MR suggests that it related to factors related to later systemic adaptations, even though we cannot know to what extent these loci actually reflect target genes. Genes that contain GR-binding loci are also found to be present in the putative pathway of stress reactivity (Figure 5).

We do not know whether the differentially expressed genes are subject to genetic regulation. In this regard, it will be of considerable interest to further study the genes that have been linked to psychiatric disorders, as genetic variation may, in fact, lead to abnormal expression of the genes we identified. It will also be of interest to study epigenetic regulation of the genes of interest and gene-environment interactions.[55-58]

Given that we assessed gene expression levels in the healthy brain, it is challenging to interpret the differences in high and low expression levels and the meaning in diseased brains. High expression levels of the genes in the stress network do not necessarily mean that stress sensitivity is a result of the high gene expression *per se*. It might be the ability to regulate neurobiological processes via direct neurotransmitter and receptor signaling or the ability to indirectly regulate changes in gene expression.[59]

Another limitation of this study is that the low number of samples in some brain regions did not allow the analysis of differential expression within these regions. For example, the precuneus and the angular gyrus were underrepresented in the AHBA (n = 7 for both regions), but harbored great changes according to the fMRI signal. However, there were sufficient brain samples available from the AHBA to analyze brain regions vulnerable to stress altogether. Furthermore, the six donors were five males and one female. It is important to take donor's sex effect into account, since there is a sex difference in the development and symptoms of stress-related diseases.[60, 61] Therefore, we checked whether gene expression levels were different for the female donor compared to the male donors. We did not find gender effects of gene expression levels of the differentially expressed genes. To maximize the number of samples, we decided to include the female donor in our analyses. It has to be taken into account, however, that the outcome of the performed task might be different across the genders.[62] This implies that our results cannot be generalized over the whole population, but are rather most reflective for males. Moreover, brain regions differentially activated by acute stress are specific for the emotion processing task. Therefore, we might have missed some relevant brain

structures, and thus genes, that might have become active during another task under stressful conditions.

To our knowledge, this is the first study to map gene expression atlases to task-based fMRI data in order to identify the molecular mechanisms underlying human stress reactivity in relation to the risk to develop psychiatric disorders. Here, we show that this method can aid in disentangling the molecular underpinnings of specific tasks and traits. We showed that genes possibly underlying stress reactivity are also associated with neuronal cell type markers (e.g. VIP expressing inhibitory neurons and glutaminergic excitatory neurons), stress-related disease, GR responsiveness and HPA-axis activity. We identified several neuropeptides and receptors as important players. These identified systems are not only important to understand the underlying mechanisms of stress vulnerability, but can also be used to develop new drug targets. Therefore, identification of novel drug targets involved in stress vulnerability would be of great interest for the development of new therapies in stress-related psychopathology.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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FIGURES / LEGENDS

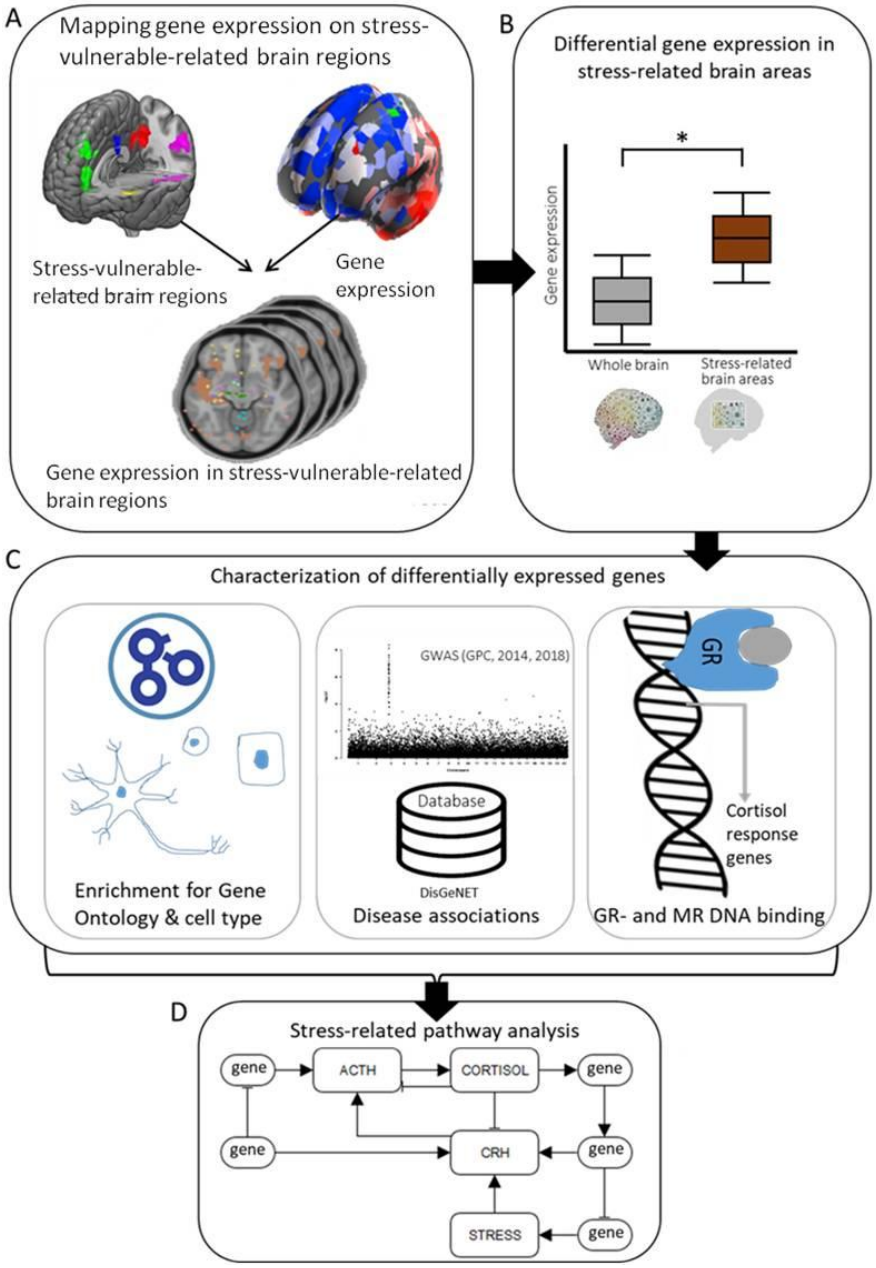


Figure 1 Study overview. (A) Brain regions vulnerable to stress (= stress network) during an emotion processing task were assessed in an fMRI study. The fMRI data was mapped to the AHBA resulting in an overlay of the fMRI and gene expression data. (B) With this overlay, differential gene expression between the brain regions vulnerable to stress and the rest of the cerebrum were assessed. (C) Differentially expressed genes were consequently characterized by identifying enrichment for gene ontology and cell type markers, associations with stress-related diseases and enrichment for cortisol responsive genes. (D) Information provided by the previous analyses was used to build a model of a molecular pathway underlying human stress reactivity.

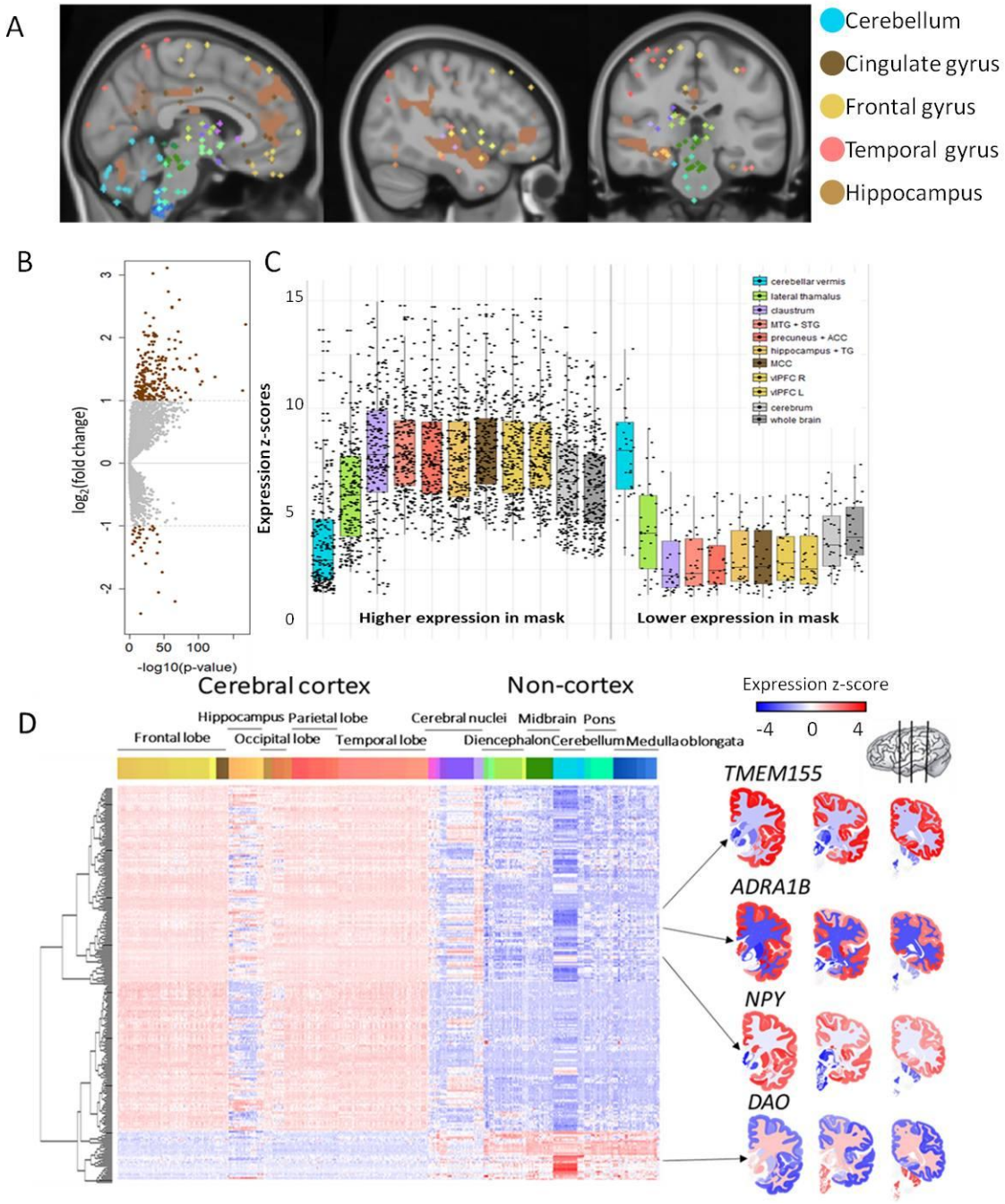


Figure 2 Differentially expressed genes in brain regions vulnerable to stress can be identified using gene expression atlases. (A) Brain regions in the stress network are present throughout the brain (including cerebellum, cingulate gyrus, frontal gyrus, temporal gyrus, and hippocampal formation). For the analysis, all regions were combined. (B) Differential gene expression was determined for the stress network compared to the rest of the cerebrum. Significant genes (BH-adjusted p-value < 0.05 & $\log_2$(fold-change) > |1|) have higher expression in the stress network. Grey dots represent non-significant genes and brown dots represent significant genes based on meta-analysis across all six

AHBA donors. (C) The box plots show the expression of the higher (left) and lower (right) expressed genes compared to the rest of the cerebrum. (D) Differentially expressed genes show high expression levels in the cortex and low expression levels in non-cortical brain regions. In the heatmap, each row represents a gene and each column represents a sample from the AHBA. On the right, coronal brain sections for the genes *TMEM155*, *ADRA1B*, *NPY* and *DAO* are presented. Colors indicates high (red) and low (blue) expression levels.

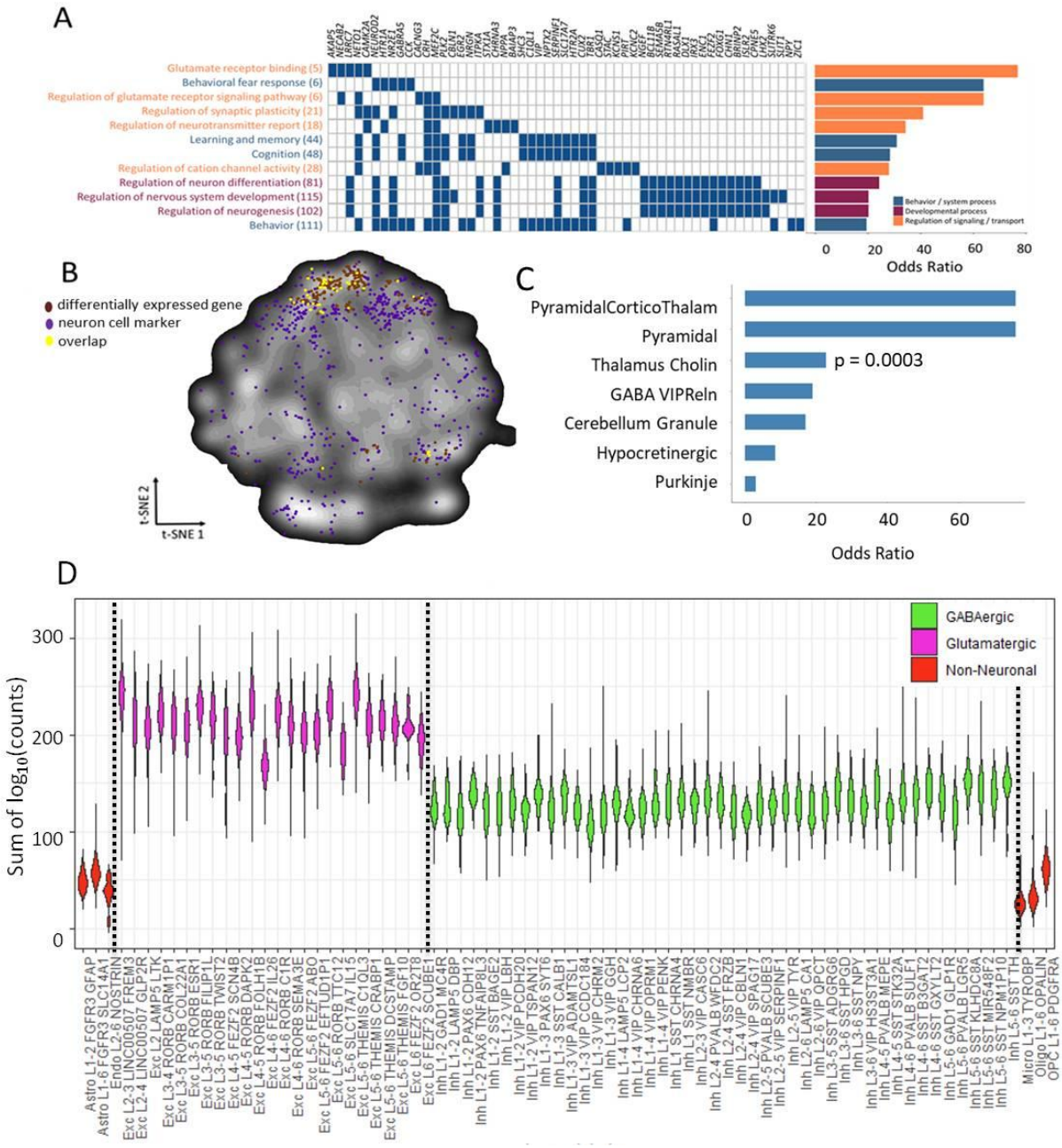


Figure 3 Functionality of differentially expressed genes in the stress network. (A) Differentially expressed genes annotated to one of the GO terms were assigned to multiple GO terms and thus involved in multiple processes. Between parenthesis, the total number of genes assigned to the GO term is depicted. On the right side of the graph, ORs are displayed per GO term. (B) Differentially expressed genes (brown), neuronal marker genes (purple) and overlapping genes (yellow) are plotted in a t-SNE plot generated using BrainScope.nl[19], where points close together represent genes with similar gene expression profiles. The differentially expressed genes show a similar profile in the t-SNE plot as neuronal cell markers (purple). (C) ORs for different neuronal subtypes. ORs were considered significant when the BH-adjusted p-value < 0.05 (*). Neuronal subtypes without a marker represented in the differentially expressed genes are not illustrated in the graph. (D) The sum of the

log_{10} values of the counts per gene is plotted for each cell cluster. Green clusters belong to GABAergic cells, purple clusters to glutamatergic cells and red clusters to non-neuronal cells.

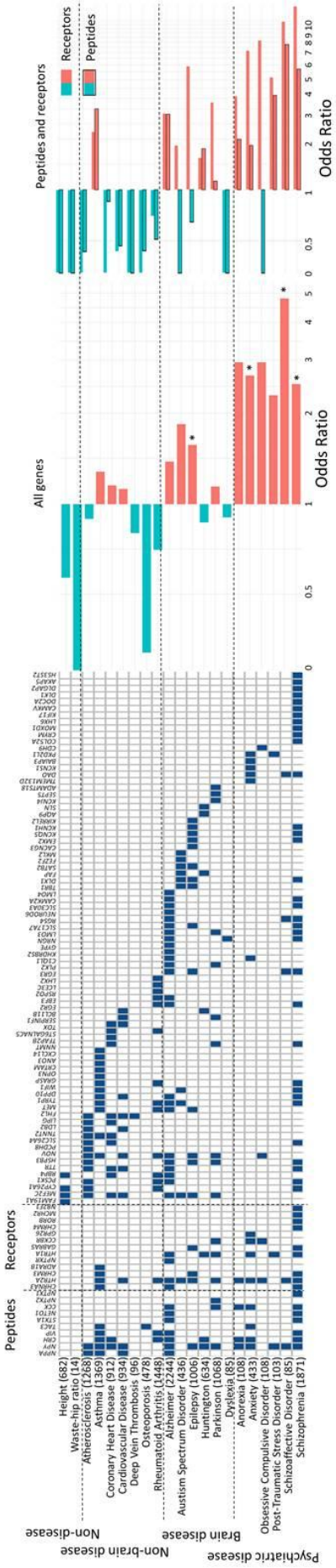

Peptides
Receptors
Height (682)
Waste-hip ratio (14)
Atherosclerosis (1268)
Asthma (1369)
Coronary Heart Disease (912)
Cardiovascular Disease (934)
Deep Vein Thrombosis (96)
Osteoporosis (478)
Rheumatoid Arthritis (1448)
Alzheimer (2244)
Austism Spectrum Disorder (436)
Epilepsy (1006)
Huntington (634)
Parkinson (1068)
Dyslexia (85)
Anorexia (108)
Anxiety (433)
Obsessive Compulsive Disorder (108)
Post-Traumatic Stress Disorder (103)
Schizoaffective Disorder (85)
Schizophrenia (1871)
Non-disease
Non-brain disease
Brain disease
Psychiatric disease
All genes
Odds Ratio
Peptides and receptors
Receptors
Peptides
Odds Ratio

Figure 4 Differentially expressed genes in the stress network and stress-related psychiatric disorders Disease risk gene enrichment was performed for the differentially expressed genes. The diseases are clustered as non-brain related disease, brain disease and psychiatric disease. As a none-disease-associated set of genes, waste-hip ratio and height were used. Numbers between the parenthesis indicate the number of genes known to be associated with the disease, based on DisGeNet. The effect size of the gene enrichment is presented at the middle part of the figure and considered significant when the BH-adjusted p-value < 0.05 (*). Blue bars mean depletion of genes, whereas red bars indicate enrichment of genes in a trait. ORs for the amount of receptors and peptides (denoted by the black borders around the bars) in the set of differentially expressed genes are depicted for every trait (shown on the right side of the graph).

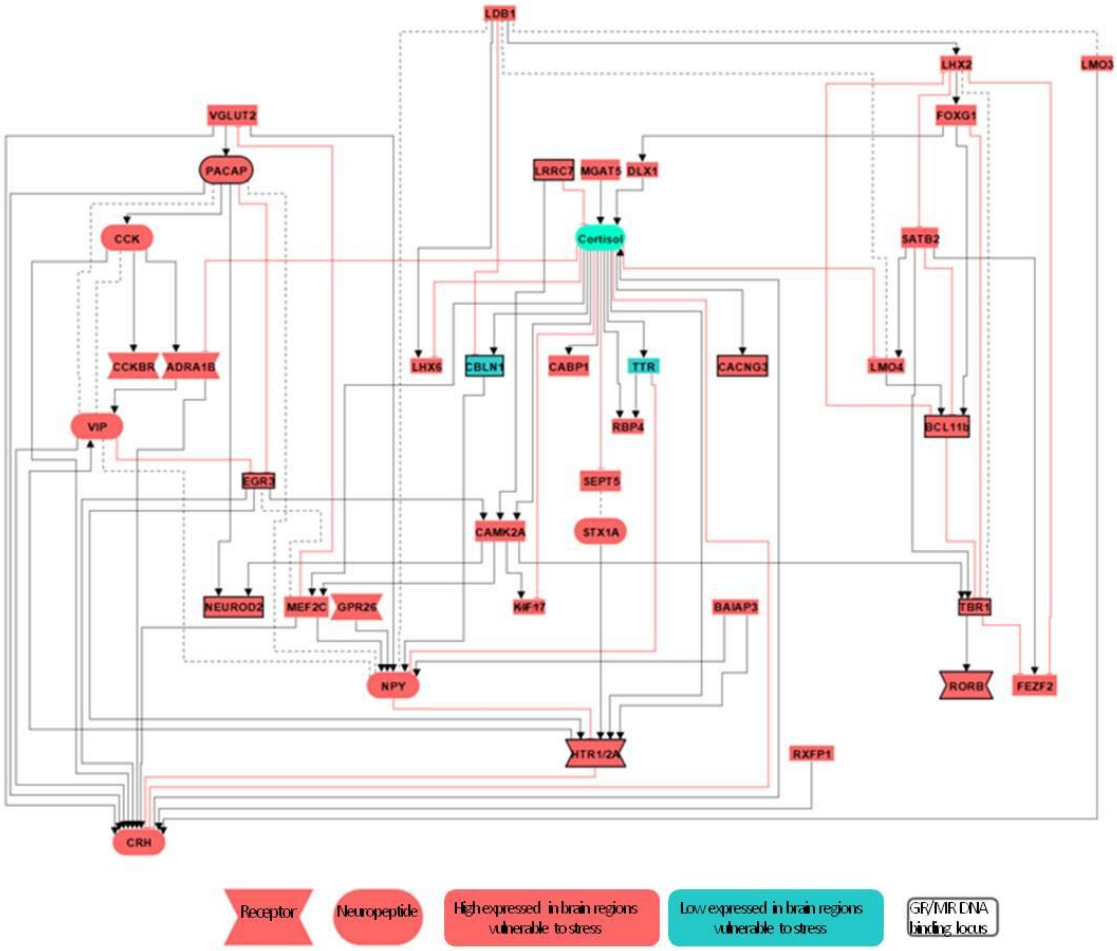


Figure 5 Putative signaling pathways as the basis of inter-individual differences in stress reactivity

Based on literature search, we assessed whether genes were known to interact with the HPA-axis and each other, resulting in a possible signaling pathway – although spatial specificity is absent from the figure. Red boxes indicate high expression levels of the differentially expressed genes in the brain regions vulnerable to stress, whereas blue boxes indicate low expression. Rounded boxes reflect neuropeptides and notched boxes reflect receptors. Squared boxes indicate the presence of a GR or MR DNA-binding loci in the vicinity of the gene. Black arrows reflect stimulation, the red t-bar indicates inhibition and the dashed line reflects binding or interaction of proteins.

Supplementary text – Sensitivity Checks

We performed sensitivity analysis to strengthen the evidence for our findings.

Differentially expressed genes in the brain regions vulnerable to stress

We identified the gene expression signatures of the stress vulnerable brain regions with altered stress-induced activity by determining which genes are differentially expressed in the brain regions vulnerable to stress compared to the rest of the cerebrum, using a meta-analysis approach. As a sensitivity analysis, we next selected genes that were differentially expressed (BH-adjusted $p < 0.05$ and $\log_2$(fold-change) > |1|) in at least five out of the six donors. This more stringent approach resulted in a smaller set of 63 differentially expressed genes (Figure S1). The smaller set of 63 genes was entirely contained within the larger set of 261 genes obtained by the meta-analysis, therefore we choose to perform subsequent analyses on the larger geneset.

The differentially expressed genes in the brain regions vulnerable to stress showed an opposite expression pattern in the cerebellum compared to the rest of the brain (Figure 2B), supporting our approach of excluding the cerebellum from our analysis (see methods).

To assess whether our results are driven by the higher representation of cerebral cortex samples in the brain regions vulnerable to stress (109 out of 127; 91%) compared to the rest of the brain (1,950 out of 3,225; 60%), we identified differentially expressed genes within each anatomically-defined region (hippocampal formation, cerebellum, frontal gyrus, temporal gyrus and cingulate gyrus) separately (Table S2). We observed an overlap (32 genes) between the non-cortex specific regions analysis compared to analysis on the whole cerebrum, suggesting that the differentially expressed genes are not only a cortical brain effect but reflect differential stress reactivity *per se.* Due to the small sample size, it was not possible to assess differential gene expression between brain regions vulnerable to stress compared to the rest of the cerebrum within each anatomically-defined region (Table S3).

Functionality and cell-type specificity of differentially expressed genes in the brain regions vulnerable to stress

To control for the case that the results of the GO term enrichment analysis are solely driven by genes already known to be associated with stress-related disorders, the analysis was also performed after exclusion of 58 genes associated to stress related disorders. We found still very similar results, indicating that differentially expressed genes in the brain regions vulnerable to stress are predominantly involved in genes relevant for stress-related diseases but not in non-brain-related disorders and traits.

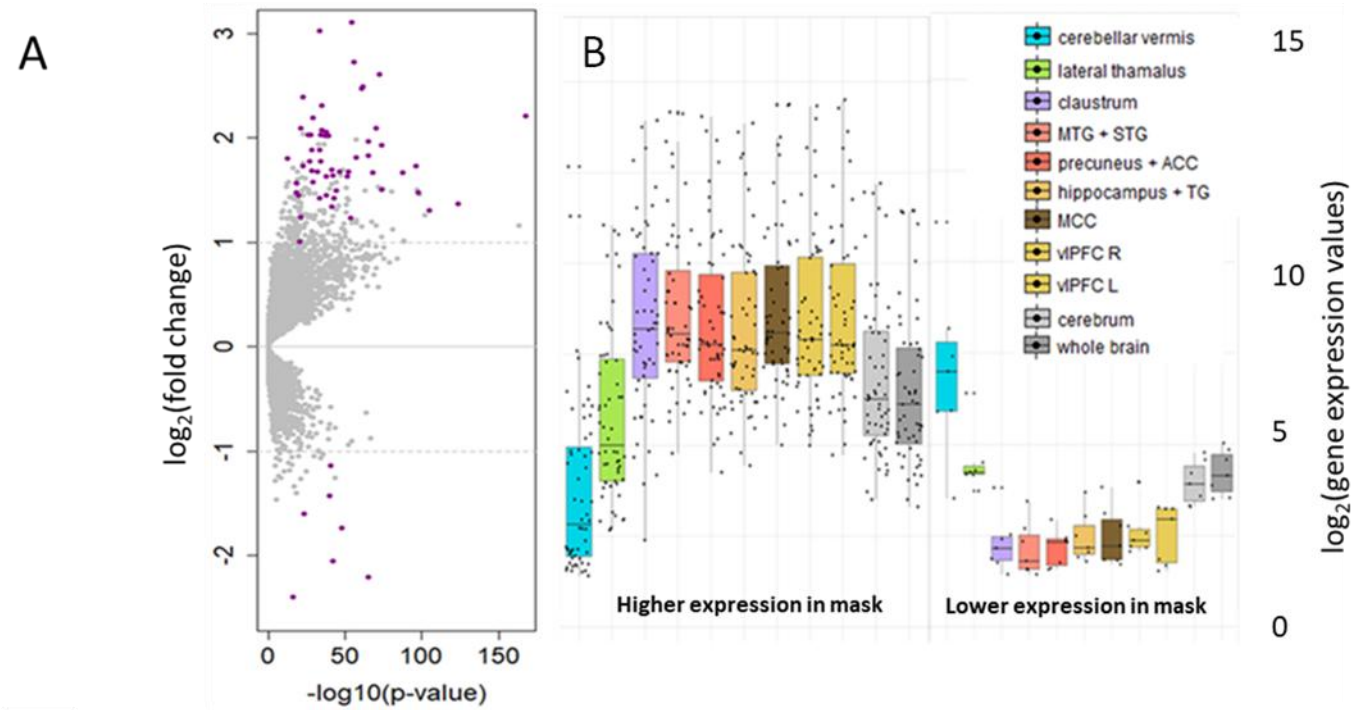
Table S2 Differentially expressed genes in the cerebellum and hippocampus

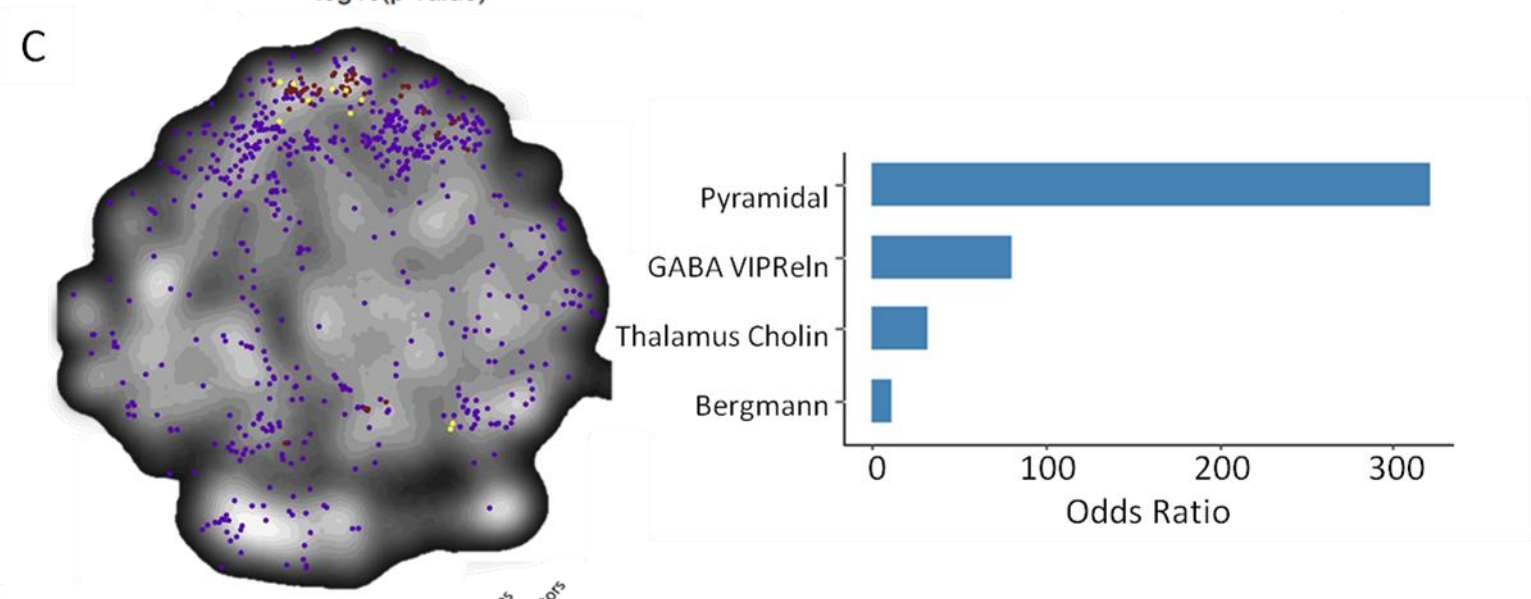
	Gene	*BH-adjusted p-value*	*Log_2(FC)*
Cerebellum (5 samples inside and 78 samples outside the stress-vulnerability related brain regions)	*SLN*	$1.7*10^{-3}$	1.13
	CCDC155	$1.3*10^{-3}$	1.01
	SEMA3C	$4.0*10^{-3}$	1.01
	NTNG1	$1.7*10^{-3}$	-1.18
	CHRNB3	$2.1*10^{-3}$	-1.35
	SHB	$6.0*10^{-4}$	-1.40
Hippocampus (19 samples inside and 26 samples outside the stress-vulnerability related brain regions)	*ZMAT4*	$1.04*10^{-10}$	1.30
	SLC17A6	$1.6*10^{-4}$	1.11
	PACAP	$2.35*10^{-5}$	1.05
	IGSF3	$1.01*10^{-7}$	1.03
	DMKN	$5.07*10^{-9}$	-1.12
	IQGAP3	$4.3*10^{-3}$	-1.15
	SCGN	$5.57*10^{-9}$	-1.48

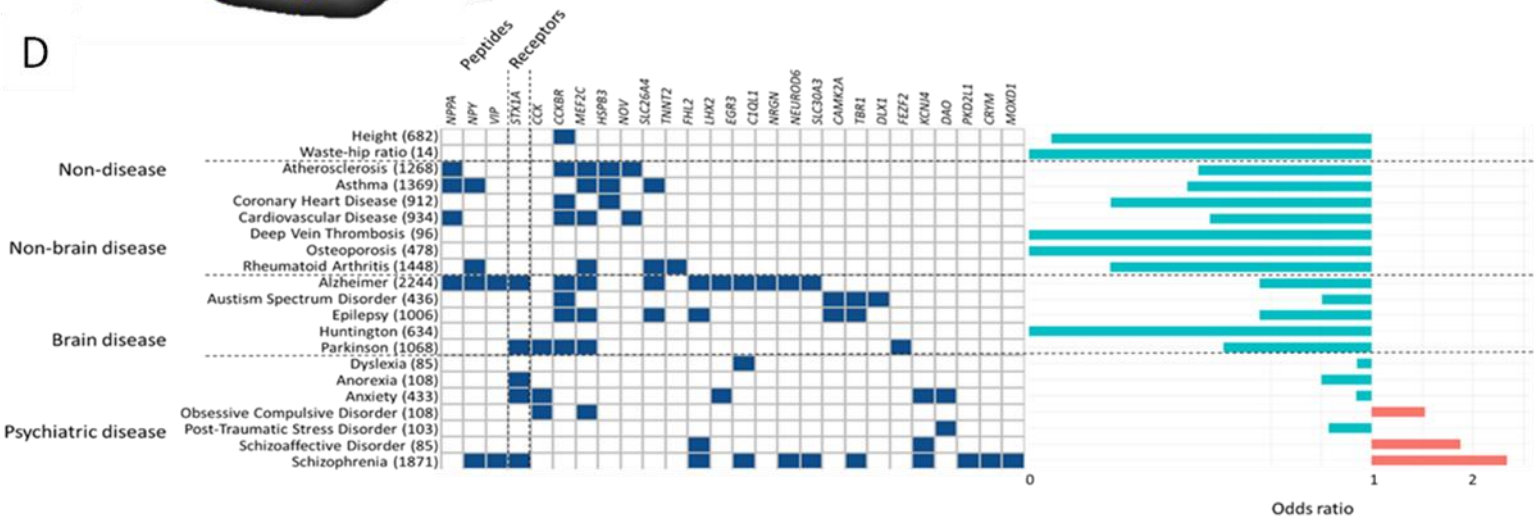
Table S3 Distribution of samples of the Allen Human Brain Atlas

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6	*Not in mask*
Superior frontal gyrus	4	6	1	1	3	3	3
Middle frontal gyrus	1	1	1	0	1	1	5
Inferior frontal gyrus	1	3	0	1	0	0	7
Parolfactory gyrus	0	1	0	0	0	0	1
Superior rostral gyrus	1	1	0	0	0	0	1
Supramarginal gyrus	0	1	0	1	2	0	4
Angular gyrus	0	0	1	0	1	1	4
Precuneus	1	0	1	0	0	1	5
Superior temporal gyrus	4	2	1	1	0	2	5
Middle temporal gyrus	5	1	1	0	0	0	4
Heschl's gyrus	0	0	0	1	0	0	1
Transverse gyrus	0	0	0	0	1	0	1
Planum temporale	2	0	0	1	0	0	1
Planum polare	1	1	2	2	1	1	1
Cuneus	2	0	0	0	0	0	5
Anterior cingulate gyrus	0	3	1	0	4	2	2
Parietal cingulate gyrus	0	0	1	2	1	1	4
Hippocampal formation	5	3	2	4	2	3	26
Claustrum	0	1	1	0	1	0	0
Lateral group of nuclei	0	3	0	1	1	0	0
Cerebellum	3	2	0	0	0	0	78

Red = <2 samples in the donors
Orange = <2 samples outside the mask
Green = comparison was possible, but effect sizes were too small


A
log2(fold change)
-log10(p-value)
B
cerebellar vermis
lateral thamalus
claustrum
MTG + STG
precuneus + ACC
hippocampus + TG
MCC
vlPFC R
vlPFC L
cerebrum
whole brain
Higher expression in mask
Lower expression in mask
log2(gene expression values)


C
Pyramidal
GABA VIPReln
Thalamus Cholin
Bergmann
Odds Ratio


D
Peptides
Receptors
NPPA
NPY
VIP
STX1A
CCK
CCKBR
MEF2C
HSPB3
NOV
SLC26A4
TNNT2
FHL2
LHX2
EGR3
C1QL1
NRGN
NEUROD6
SLC30A3
CAMK2A
TBR1
DLX1
FEZF2
KCNJ4
DAO
PKD2L1
CRYM
MOXD1
Height (682)
Waste-hip ratio (14)
Atherosclerosis (1268)
Asthma (1369)
Coronary Heart Disease (912)
Cardiovascular Disease (934)
Deep Vein Thrombosis (96)
Osteoporosis (478)
Rheumatoid Arthritis (1448)
Alzheimer (2244)
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Epilepsy (1006)
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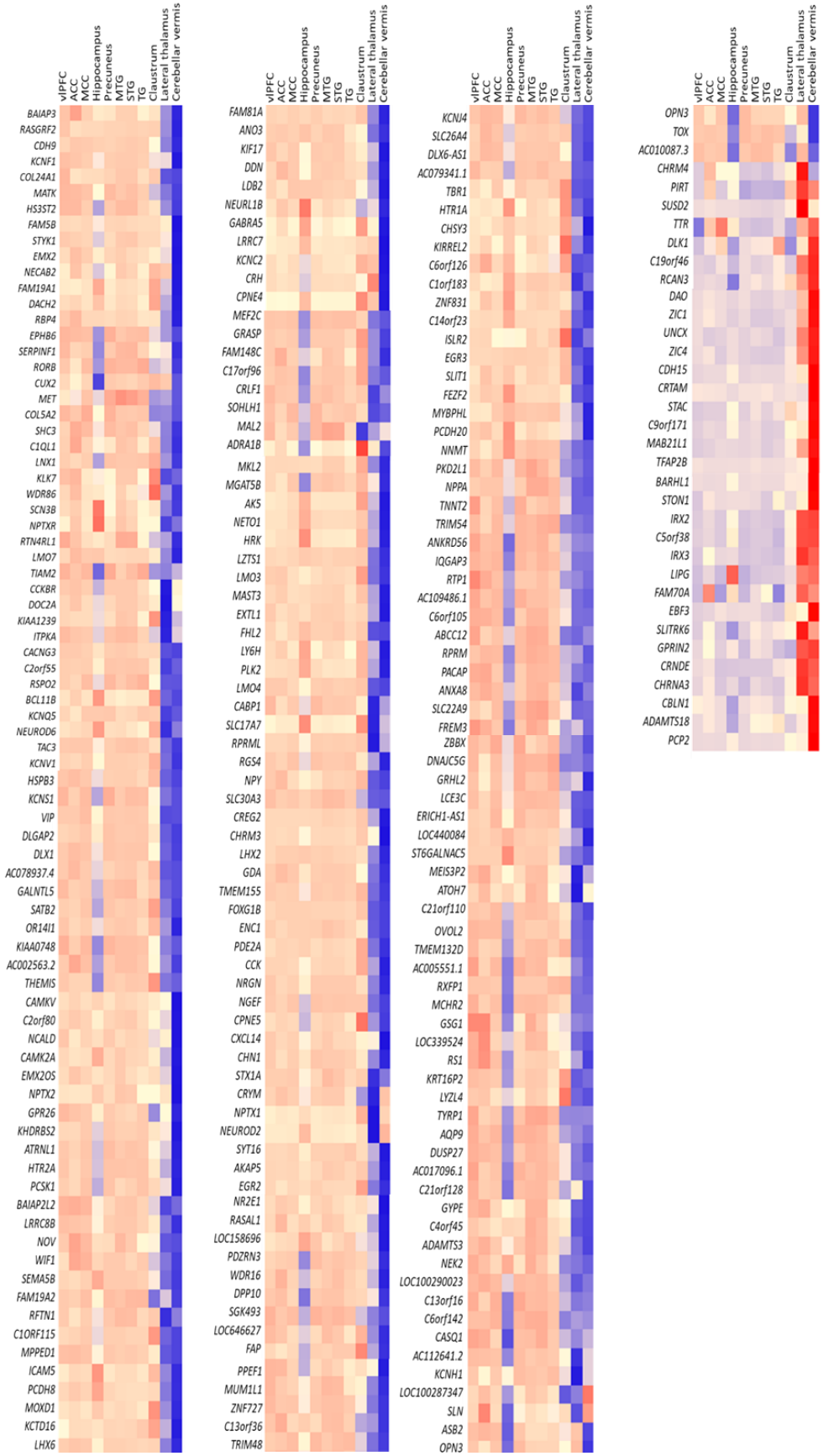
Parkinson (1068)
Dyslexia (85)
Anorexia (108)
Anxiety (433)
Obsessive Compulsive Disorder (108)
Post-Traumatic Stress Disorder (103)
Schizoaffective Disorder (85)
Schizophrenia (1871)
Non-disease
Non-brain disease
Brain disease
Psychiatric disease
Odds ratio

Figure S1 Using a stricter method of selecting differentially expressed genes results in similar outcome as using a meta-analysis. (A) Differential gene expression was determined for the stress network compared to the rest of the cerebrum for each of the six donors separately. Genes showing significance (BH-adjusted p-value < 0.05 & $\log_2$(fold-change) > |1|) in at least 5 out of the 6 donors were selected and are shown in purple in the volcano plot of the meta-analysis. Grey dots represent non-significant genes. The box plots on the right show the expression of the individual differentially expressed genes relative to the average gene expression in individual brain regions. (B) Differentially expressed genes (brown), neuronal marker genes (purple) and overlapping genes (pink) are plotted in a t-Distributed Stochastic Neighbor Embedding (t-SNE) plot acquired from BrainScope.nl[19], visualized in a heatmap of genes, where close points represent genes with similar gene expression profiles. ORs for different neuronal subtypes are presented. Neuronal subtypes without a marker represented in the differentially expressed genes are not illustrated in the graph. No significant enrichment for neuronal markers was observed. (C) Disease-risk gene enrichment was performed for the differentially expressed genes. The diseases are clustered as non-brain related disease, brain disease and psychiatric disorders. As a none-disease-associated set of genes, waste-hip ratio and height were used. The numbers between the parenthesis indicate the amount of genes known to be associated with the disease based on DisGeNet. The effect size of the gene enrichment is presented on the right side of the figure, though none of them was significant.


vlPFC
ACC
MCC
Hippocampus
Precuneus
MTG
STG
TG
Claustrum
Lateral thalamus
Cerebellar vermis
BAIAP3
RASGRF2
CDH9
KCNF1
COL24A1
MATK
HS3ST2
FAM5B
STYK1
EMX2
NECAB2
FAM19A1
DACH2
RBP4
EPHB6
SERPINF1
RORB
CUX2
MET
COL5A2
SHC3
C1QL1
LNX1
KLK7
WDR86
SCN3B
NPTXR
RTN4RL1
LMO7
TIAM2
CCKBR
DOC2A
KIAA1239
ITPKA
CACNG3
C2orf55
RSPO2
BCL11B
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NEUROD6
TAC3
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GALNTL5
SATB2
OR14I1
KIAA0748
AC002563.2
THEMIS
CAMKV
C2orf80
NCALD
CAMK2A
EMX2OS
NPTX2
GPR26
KHDRBS2
ATRNL1
HTR2A
PCSK1
BAIAP2L2
LRRC8B
NOV
WIF1
SEMA5B
FAM19A2
RFTN1
C1ORF115
MPPED1
ICAM5
PCDH8
MOXD1
KCTD16
LHX6
FAM81A
ANO3
KIF17
DDN
LDB2
NEURL1B
GABRA5
LRRC7
KCNC2
CRH
CPNE4
MEF2C
GRASP
FAM148C
C17orf96
CRLF1
SOHLH1
MAL2
ADRA1B
MKL2
MGAT5B
AK5
NETO1
HRK
LZTS1
LMO3
MAST3
EXTL1
FHL2
LY6H
PLK2
LMO4
CABP1
SLC17A7
RPRML
RGS4
NPY
SLC30A3
CREG2
CHRM3
LHX2
GDA
TMEM155
FOXG1B
ENC1
PDE2A
CCK
NRGN
NGEF
CPNE5
CXCL14
CHN1
STX1A
CRYM
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DPP10
SGK493
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TBR1
HTR1A
CHSY3
KIRREL2
C6orf126
C1orf183
ZNF831
C14orf23
ISLR2
EGR3
SLIT1
FEZF2
MYBPHL
PCDH20
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NPPA
TNNT2
TRIM54
ANKRD56
IQGAP3
RTP1
AC109486.1
C6orf105
ABCC12
RPRM
PACAP
ANXA8
SLC22A9
FREM3
ZBBX
DNAJC5G
GRHL2
LCE3C
ERICH1-AS1
LOC440084
ST6GALNAC5
MEIS3P2
ATOH7
C21orf110
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RXFP1
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RS1
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LYZL4
TYRP1
AQP9
DUSP27
AC017096.1
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GYPE
C4orf45
ADAMTS3
NEK2
LOC100290023
C13orf16
C6orf142
CASQ1
AC112641.2
KCNH1
LOC100287347
SLN
ASB2
OPN3
OPN3
TOX
AC010087.3
CHRM4
PIRT
SUSD2
TTR
DLK1
C19orf46
RCAN3
DAO
ZIC1
UNCX
ZIC4
CDH15
CRTAM
STAC
C9orf171
MAB21L1
TFAP2B
BARHL1
STON1
IRX2
C5orf38
IRX3
LIPG
FAM70A
EBF3
SLITRK6
GPRIN2
CRNDE
CHRNA3
CBLN1
ADAMTS18
PCP2

Figure S2 Differential gene expression in the stress network. For each brain region vulnerable to stress, relative expression levels are shown. Colors indicates high (red) or low (blue) expression.

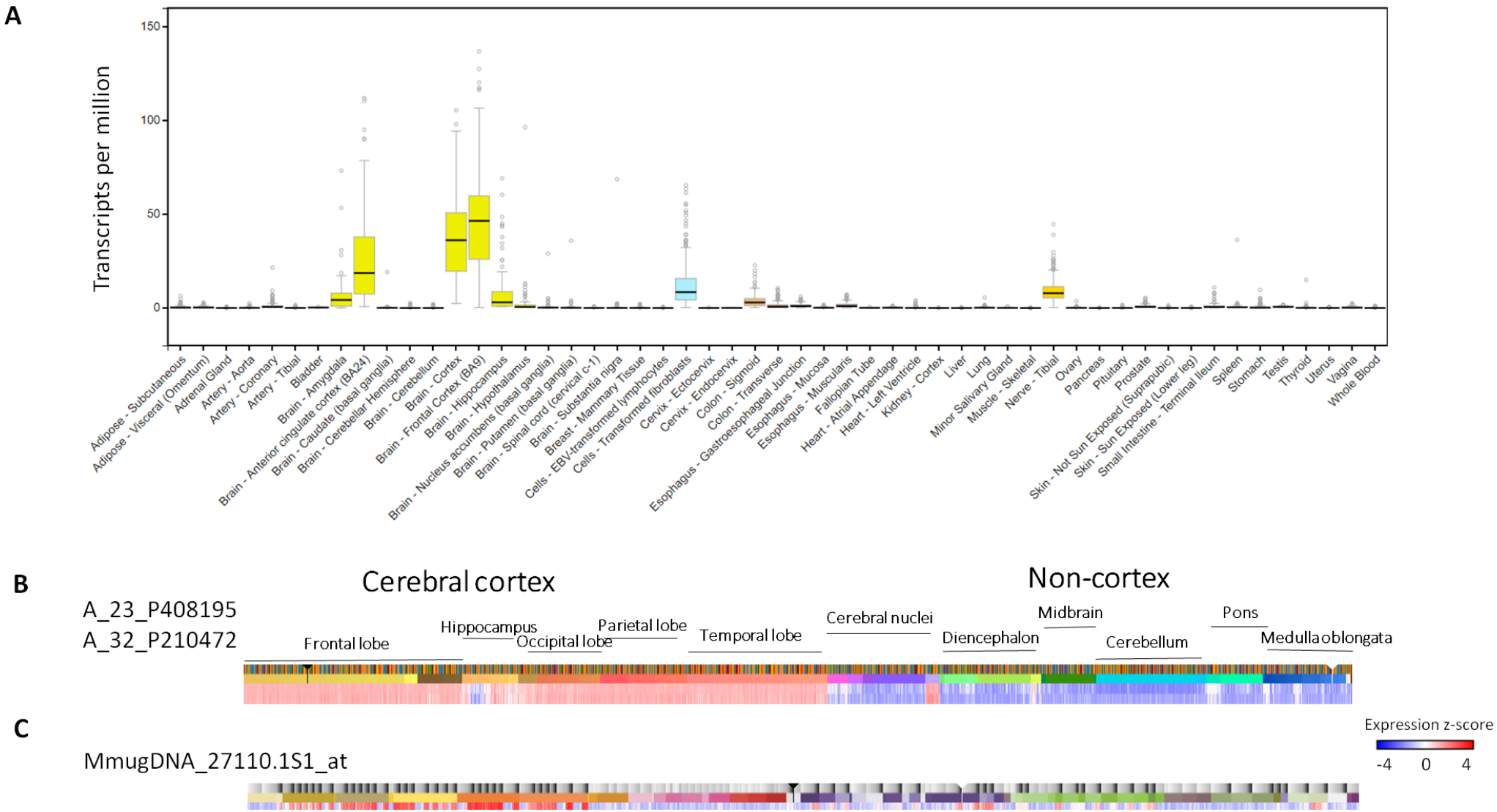
A
Transcripts per million
150
100
50
0
Adipose - Subcutaneous
Adipose - Visceral (Omentum)
Adrenal Gland
Artery - Aorta
Artery - Coronary
Artery - Tibial
Bladder
Brain - Amygdala
Brain - Anterior cingulate cortex (BA24)
Brain - Caudate (basal ganglia)
Brain - Cerebellar Hemisphere
Brain - Cerebellum
Brain - Cortex
Brain - Frontal Cortex (BA9)
Brain - Hippocampus
Brain - Hypothalamus
Brain - Nucleus accumbens (basal ganglia)
Brain - Putamen (basal ganglia)
Brain - Spinal cord (cervical c-1)
Brain - Substantia nigra
Breast - Mammary Tissue
Cells - EBV-transformed lymphocytes
Cells - Transformed fibroblasts
Cervix - Ectocervix
Cervix - Endocervix
Colon - Sigmoid
Colon - Transverse
Esophagus - Gastroesophageal Junction
Esophagus - Mucosa
Esophagus - Muscularis
Fallopian Tube
Heart - Atrial Appendage
Heart - Left Ventricle
Kidney - Cortex
Liver
Lung
Minor Salivary Gland
Muscle - Skeletal
Nerve - Tibial
Ovary
Pancreas
Pituitary
Prostate
Skin - Not Sun Exposed (Suprapubic)
Skin - Sun Exposed (Lower leg)
Small Intestine - Terminal Ileum
Spleen
Stomach
Testis
Thyroid
Uterus
Vagina
Whole Blood
B
Cerebral cortex
Non-cortex
A_23_P408195
A_32_P210472
Frontal lobe
Hippocampus
Occipital lobe
Parietal lobe
Temporal lobe
Cerebral nuclei
Diencephalon
Midbrain
Cerebellum
Pons
Medulla oblongata
C
MmugDNA_27110.1S1_at
Expression z-score
-4
0
4

Figure S3 *TMEM155* is highly expressed in the claustrum. (A) In the human body, *TMEM155* has only been identified in the brain. (B) In the human brain, *TMEM155* (measured using two probes: A_23_P408195 and A_32_P210472) is highly expressed in the cortex and claustrum. (C) The expression pattern of *TMEM155* (measured using one probe: MmugDNA_27110.1S1_at) in the macaque brain resembles the human expression pattern.