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ORIGINAL ARTICLE

Molecular indicators of stress-induced neuroinflammation in a mouse model simulating features of post-traumatic stress disorder

S Muhie[1,2], A Gautam[3], N Chakraborty[1], A Hoke[1], J Meyerhoff[1], R Hammamieh[3] and M Jett[3]

A social-stress mouse model was used to simulate features of post-traumatic stress disorder (PTSD). The model involved exposure of an intruder (male C57BL/6) mouse to a resident aggressor (male SJL) mouse for 5 or 10 consecutive days. Transcriptome changes in brain regions (hippocampus, amygdala, medial prefrontal cortex and hemibrain), blood and spleen as well as epigenome changes in the hemibrain were assayed after 1- and 10-day intervals following the 5-day trauma or after 1- and 42-day intervals following the 10-day trauma. Analyses of differentially expressed genes (common among brain, blood and spleen) and differentially methylated promoter regions revealed that neurogenesis and synaptic plasticity pathways were activated during the early responses but were inhibited after the later post-trauma intervals. However, inflammatory pathways were activated throughout the observation periods, except in the amygdala in which they were inhibited only at the later post-trauma intervals. Phenotypically, inhibition of neurogenesis was corroborated by impaired Y-maze behavioral responses. Sustained neuroinflammation appears to drive the development and maintenance of behavioral manifestations of PTSD, potentially via its inhibitory effect on neurogenesis and synaptic plasticity. By contrast, peripheral inflammation seems to be directly responsible for tissue damage underpinning somatic comorbid pathologies. Identification of overlapping, differentially regulated genes and pathways between blood and brain suggests that blood could be a useful and accessible brain surrogate specimen for clinical translation.

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INTRODUCTION

Stress-induced maladaptive responses are major health problems affecting significant proportions of emergency responders, soldiers, victims of abuse, and survivors of accidents, violence and natural disasters. Varied types and durations of traumatic exposure (single or episodic, acute or chronic stressful events) have been shown to induce post-traumatic stress disorder (PTSD) in human[1,2] and PTSD-like phenotypes in animal models.[3–6]

Stress-induced PTSD is a complex, poorly understood and growing problem. In its chronic form, PTSD includes multisystem disorders with comorbidities encompassing both physical and psychiatric maladies. Despite being prevalent, robust diagnoses and prognoses of PTSD are limited and optimal therapeutic targets are lacking. The primary challenge is the difficulty of detecting and treating early stages of the disorder, as well as predicting clinical courses and outcomes. Even known risk factors such as genetic, epigenetic, endocrine and demographic factors (sex, education, income and social status), prior adverse life events, or a history of early trauma, do not effectively predict disease severity or chronicity.[7] Moreover, the widely practiced self-reported and physician opinion-based diagnoses lack objectivity.

Because targets for early intervention remain elusive, a better understanding of the molecular underpinnings of PTSD pathogenesis is needed to meet the challenges of disease prognosis, diagnosis and treatment. This includes identifying molecular alterations in signaling pathways and processes that underlie the response to PTSD triggering stress. Differentially altered pathways and processes, in conjunction with pre-existing genetic and epigenetic factors, are likely to mediate stress-induced pathologies, and contribute to the onset, development, severity and persistence of the disorder.

Increasing evidence highlights the involvement of persistent dysfunctions of immune response pathways in psychiatric and psychopathological diseases,[8] including PTSD.[9] Particularly, stress-induced systemic inflammation has been associated with learning and memory impairments, leading to cognitive and behavioral deficits.[10] For example, stress-activated nuclear factor-κB (NF-κB; and interleukin (IL)-1β) signaling has been reported to inhibit hippocampal neurogenesis potentially leading to behavioral disorder.[11] In addition to transcription-mediated modulation, long-lasting molecular mechanisms such as epigenetic modifications have been implicated in mediating the persistent impact of stress-induced inflammation on cognition and behavior.[10] Despite these promising findings, most studies attempting to connect the effect of inflammatory molecules with cognition and behavioral deficits are limited by their focus on single time point observations.

A temporal delineation of PTSD development from the nuclear stressor event to altered molecular events is critical for identifying prognostic markers. However, availabilities of molecular data directly connecting clinical phenotypes to signaling pathways and cellular processes are currently limited. Furthermore, molecular

[1]The Geneva Foundation, Frederick, MD, USA; [2]Advanced Academics Programs, Krieger School of Arts & Sciences, Johns Hopkins University, Baltimore, MD, USA and [3]Integrative Systems Biology, US Army Center for Environmental Health Research, Frederick, MD, USA. Correspondence: Dr M Jett, Integrative Systems Biology, US Army Center for Environmental Health Research, 568 Doughten Drive, Fort Detrick, Frederick, MD 21702-5010, USA.
E-mail: marti.jett-tilton.civ@mail.mil
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mechanisms and outcome predictors determining the temporal trajectories of stress-induced pathologies leading to PTSD are yet to be elucidated. In this study, we examined longitudinal multi-omics alterations across tissues and behaviorally relevant brain regions and blood from a social-stress mouse model simulating features of PTSD. Such approaches have the potential to provide a better understanding of molecular alterations leading to PTSD. First, temporal trajectories should help to delineate the type and timing of molecular events underlying adaptive versus maladaptive reactions to traumatic stress. Second, a shift in focus from a single cytokine or neuronal factor to networks of molecular interactions and their predicted activation status should provide molecular mechanisms contributing to PTSD, as well as the molecular milieu in which comorbidities are maintained.

The social-stress model, used in this study, involved random exposure of an intruder male C57BL/6 mouse to a trained aggressor resident male SJL mouse. The subject C57BL/6 mouse was later exposed to trauma reminders (aggressor barrier avoidance tests) at various post-trauma intervals.[12] Such random exposure to a conspecific aggressor was found to elicit important behavioral features reported to occur in PTSD patients such as avoidance of trauma reminders (marked avoidance of aggressor barrier), jumping during handling (increased vigilance) or freezing (social withdrawal) and impaired cognition.[12–14] Using this model, we profiled transcriptome alterations in brain regions (hippocampus, amygdala, medial prefrontal cortex and hemibrain), blood and spleen as well as DNA methylome changes in the hemibrain at multiple time points.

Transcripts with overlapping expression profiles among brain, blood and spleen showed activated inflammation, as well as inhibited neurogenesis and impaired synaptic plasticity at longer post-trauma delays. Activation status of signaling pathways at multiple time points demonstrated the systemic effect of inflammation on neurogenesis and on cognition (learning and memory), while also accounting for the chronic pain and physical complaints of PTSD patients. Identification of overlapping differentially expressed genes (DEGs) and pathways between blood and brain suggests that blood can be used as an accessible brain surrogate sample for clinical translation.

Our findings suggest that stress-activated inflammatory responses play a critical role in the development and maintenance of PTSD-like disorders and comorbid conditions. Hence, diagnosis and treatment to alleviate stress-induced PTSD might eventually include strategies for addressing the inflammatory problem to significantly improve patients' quality of life.

MATERIALS AND METHODS

Mouse model and behavioral evaluations

Details about the mouse model and PTSD-related behavioral characterizations of the model have been reported earlier.[12,13] Briefly, naive C57BL/6 male mice (8–10 weeks old) were single-housed, and mice ($N = 5$ per group) were randomly assigned to aggressor-exposed (Agg-E) and cage-control (C-ctrl) groups for each time point. Each Agg-E mouse was kept in a mesh box inside the cage of a trained, highly aggressive, resident SJL mouse for 6 h/day for either 5 (T5) or 10 (T10) consecutive days. Each day, a different aggressor mouse was used, with the Agg-E mouse subjected to two to three direct exposures to the aggressor mouse, at random, for 1 min or 10 strikes, whichever came first. This set-up added both uncontrollability and randomness factors to the social defeat stress (to simulate some aspects of PTSD). The defeated mice were inspected before and after each defeat session for scratches or wounding to exclude any mouse with skin-deep wounds from the experiment. Control (never exposed to aggressor) mice were identically housed for the 6-h period in a separate room (for the same number of days). An hour before cervical dislocation, each mouse was tested for avoidance behavior by measuring the time spent in an 'interaction zone' around a caged aggressor, physically separated by a plastic divider with holes, which allowed visual, olfactory and auditory contact during the avoidance behavioral assessment. Summaries of experimental conditions and collected tissues are shown in Supplementary Figure 1 and Supplementary Table 1. Experiments and procedures on mice were approved by the local (Walter Reed Army Institute of Research) Institutional Animal Care and Use Committees, and were conducted in strict accordance with AAALAC Guidelines.

Y-maze test

The spontaneous alternation task was performed using the Y-maze apparatus with three identical arms that were 12.7 cm wide, 20.32 cm long and evenly separated at 120° from each other (which were arbitrary called arms A, B and C). The Y-maze test was performed 1 day after T10 Agg-E (or control), and behaviors were observed for 7 min, following 1-min acclimation to the maze. Combinations of three unique arms such as 'ABC', 'BAC' and 'BCA' were used as spatial parameters, and percent-alternation rates were calculated using the number of possible alternations:

$$\%\ \text{of alternation rate} = \frac{\text{number of alternations (combination of unique arms)} \times 100}{\text{total number of possible alternations (total number of possible arms)}}$$

Sample collection

Mice were killed by cervical dislocation, alternating between Agg-E and C-ctrl mice to control for time of day effects. Blood from each mouse was collected by percutaneous cardiac puncture and stabilized with the PAXgene Blood RNA solution (Qiagen, Valencia, CA, USA). The spleens were also collected to identify more stable (less transient) overlapping molecular alterations with (the more dynamic) blood. Brain regions, hemibrain, hippocampus (HC), amygdala (AY) and medial prefrontal cortex (MPFC), were dissected following a previously reported procedure.[14]

RNA isolation

Total RNA was isolated from homogenized brain regions and spleen in the TRIzol reagent (Invitrogen, Thermo Fischer Scientific, Carlsbad, CA, USA) and from blood using the PreAnalytiX PAXgene Blood RNA kit (Qiagen) following the manufacturers' protocols. RNA was evaluated using the NanoDrop spectrophotometer (Thermo Fischer Scientific) and Tapestation2200 (Agilent Technologies, Santa Clara, CA, USA).

Assays

Expression arrays, DNA methylation (tiling) arrays and validation assays. Expression microarrays for all tissues were performed using Agilent's Genome-wide Mouse Expression arrays (GE_4x44Kv2 two-color), whereas DNA methylation tiling arrays for hemibrain were carried out using Agilent's Mouse CpG-Island arrays (2×105 K), both following the manufacturer's protocol. Hybridized microarray slides were scanned using Agilent's G2505C-US09493743-Scanner, and images were processed using the default set-up of Agilent's Feature Extraction Software v10.7.

Processed data sets were filtered to exclude probes with missing values in more than one sample, and quantile normalized using Limma, R-Package (www.bioconductor.org). Normalized data sets were analyzed/compared for stress effects using the moderated *T*-test of the Limma package.

DEGs from blood were validated by measuring plasma-protein levels. Blood samples were collected in 50 µl of buffered sodium citrate (0.105 M —3.2%) and centrifuged to separate plasma, which was snap-frozen. A measure of 70 µl of plasma was shipped to Rules-Based-Medicine, Austin, TX, USA, for Rodent-MAPv.20 antigens assay using the Luminex technology.

DEGs from hemibrain were validated using QuantiGene2.0 Multiplex-Bead-Assay (QuantiGene, Affymetrix, Sanata Clara, CA, USA), whereas DEGs from spleen were validated using nanoString transcript assay (nCounter GeneExpressionCode-Sets, NanoString Technologies, Seattle, WA, USA).

Inflammatory panel assays. Mouse-Inflammation-Panel v2 was used for AY, HC and MPFC samples according to the manufacturer's protocol (NanoString Technologies). Briefly, total RNA (100 ng) was hybridized overnight, followed by post-hybridization processing. Data sets were analyzed using the recommended settings of the nSolver-pipeline (NanoString Technologies) for background subtraction, quality control on samples/lanes and the downstream data analyses as follows: normalization, statistical significance testing and fold change (FC) calculations. Transcripts that passed $P < 0.1$ and $FC > 1.5$ in each region (AY, HC or MPFC) at one or more time points (T5R1, T10R1, T5R10 and T10R42 except

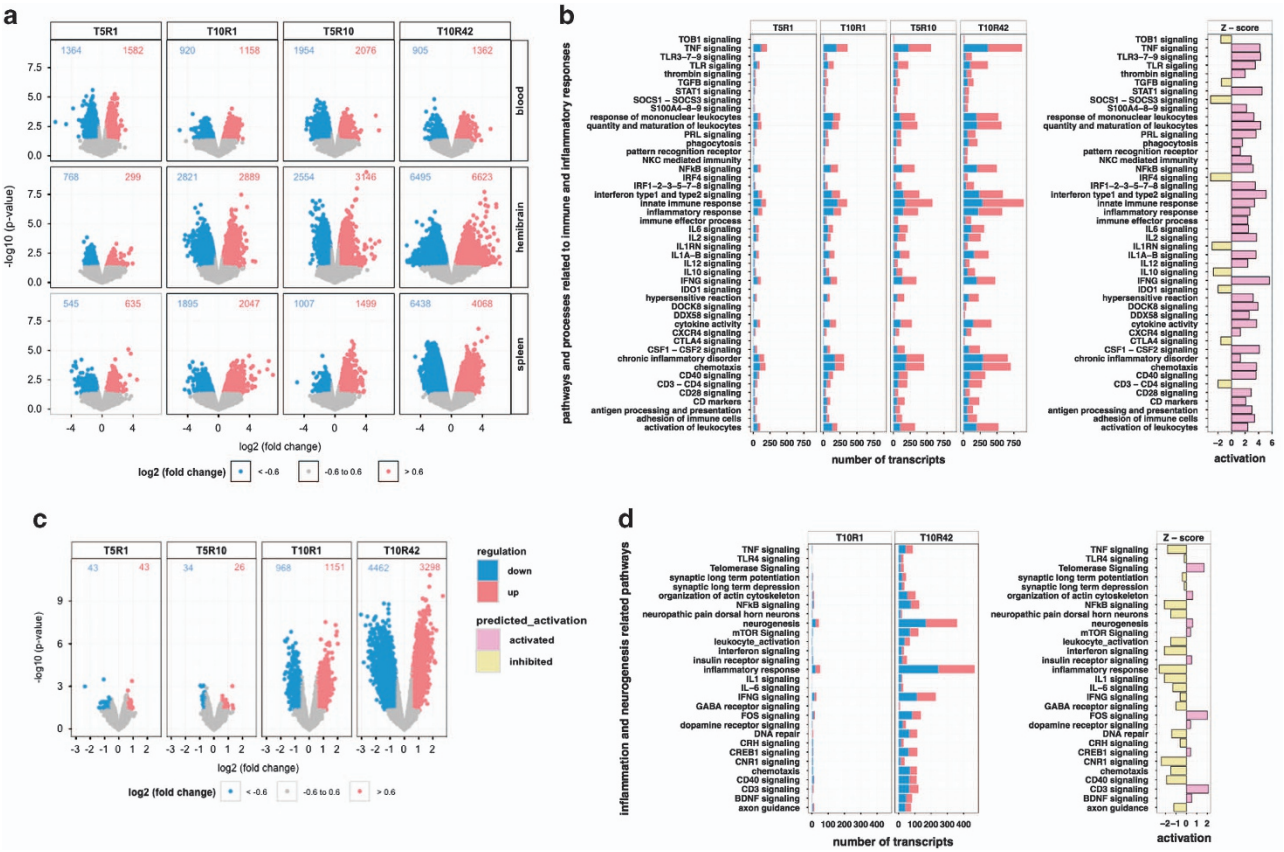

Figure 1. (**a**) Differentially expressed genes (DEGs) in blood, hemibrain and spleen of trauma-exposed C57BL/6 male mice across different time points. DEGs (blue and red dots) from each tissue at each time point were identified using moderated *T*-test at $P < 0.05$ and fold change (FC) > 1.5 filters. The scatter plots summarized the number and directions of DEGs from each tissue at each time point including their significance levels and effect sizes (in log FCs). The gene symbols, *P*-values and $\log_2$ FCs for the top DEG from each tissue and time point are given in Supplementary Table 2. (**b**) Pathways and processes related to inflammatory responses and innate immunity. These were identified using initially unbiased enrichments for overlapping DEGs across tissues. Predicted activation states (purple and yellow bars) were colored based on predicted activation or inhibition *z*-scores of pathways or processes enriched at day 42 after the 10-day aggressor exposure session (T10R42), which possibly corresponds to persistent post-traumatic stress disorder in human. The identity, FC and significance levels of genes associated with each of these pathways and process are given in Supplementary Table 3. (**c**) Differentially methylated promoter probes in hemibrain. Differentially methylated promoter CpG islands (blue and red dots) were identified using moderated *T*-test, $P < 0.05$, FC > 1.5. The scatter plots showed promoter regions of more genes were differentially methylated due to the longer trauma session (10-day aggressor exposure session), and even more so at the longer post aggressor exposure days (at T10R42). (**d**) Processes and pathways associated with differentially methylated probes from hemibrain at the promoter region ($q < 0.05$). Predicted activation or inhibition states of these pathways and processes were colored based on the activated or inhibited methylation *z*-scores of promoter CpGs of genes significantly associated with the corresponding pathways and processes. Promoter regions of genes significantly associated with inflammatory pathways and processes were predicted to have inhibited DNA methylation states, whereas promoter regions of genes significantly associated with neurogenesis were predicted to have activated DNA methylation pattern. (These pathways and processes were enriched using hypergeometric test with false discovery rate of $q < 0.05$.)

MPFC-T10R42, due to sample availability) were used for further analyses and downstream visualization.

Data analysis

Differentially methylated promoter regions and DEGs, as determined between Agg-E and C-ctrl mice, were identified at the four time points using the Moderated T-test in Limma (false discovery rate, $q \leqslant 0.05$) and $P \leqslant 0.05$, respectively). Time effects and trends in transcriptome changes across post-trauma days (for overlapping DEGs among brain, blood and spleen) were identified using Bayesian and temporal clustering methods.[15]

Hypergeometric test ($q < 0.05$), Bingov3.0.3 (http: apps.cytoscape.org/apps/bingo) and ClueGOv2.2.5 (http://www.cytoscape.org), Fisher's exact test (Ingenuity, Redwood, CA, USA), Network Analyst[16] and Gene Set Enrichment Analysis (http://www.broadinstitute.org/gsea) were used to determine an unbiased gene ontology and pathway enrichments of overlapping DEGs. Significant processes and pathways were graphed and/or visualized using R-programmingv3.3.0, Cytoscapev3.4 (https://cran.r-project.org/), Gephiv0.9.1 (https://gephi.org) and Ingenuity Pathways Analysis.

Activation *z*-scores for significantly enriched pathways and processes were calculated to predict activation states based on intermolecular connectors (edges).[17,18] Experimental directions of gene expression for members of the pathway or the network were compared with literature-derived activation directions (as curated in Ingenuity Pathways Analysis) for either activating or inhibiting effects (cause–effect relationship and direction of effect).[17,18]

Functional networks and bar graphs for significant pathways and processes indicating the directions and number of up- or downregulated DEGs, and activation state of the corresponding pathway or process (values and directions of z-scores), were constructed using Cytoscapev3.4.0 and ggplot2 package of R, and visualized using Gephiv0.9.1 and Rstudiov0.99.484 (https://www.rstudio.com).

RESULTS

DEGs and methylated promoter regions

DEGs from each tissue at each time were identified using a moderated *t*-test (at $P < 0.05$, and FC > 1.5). DEGs that passed

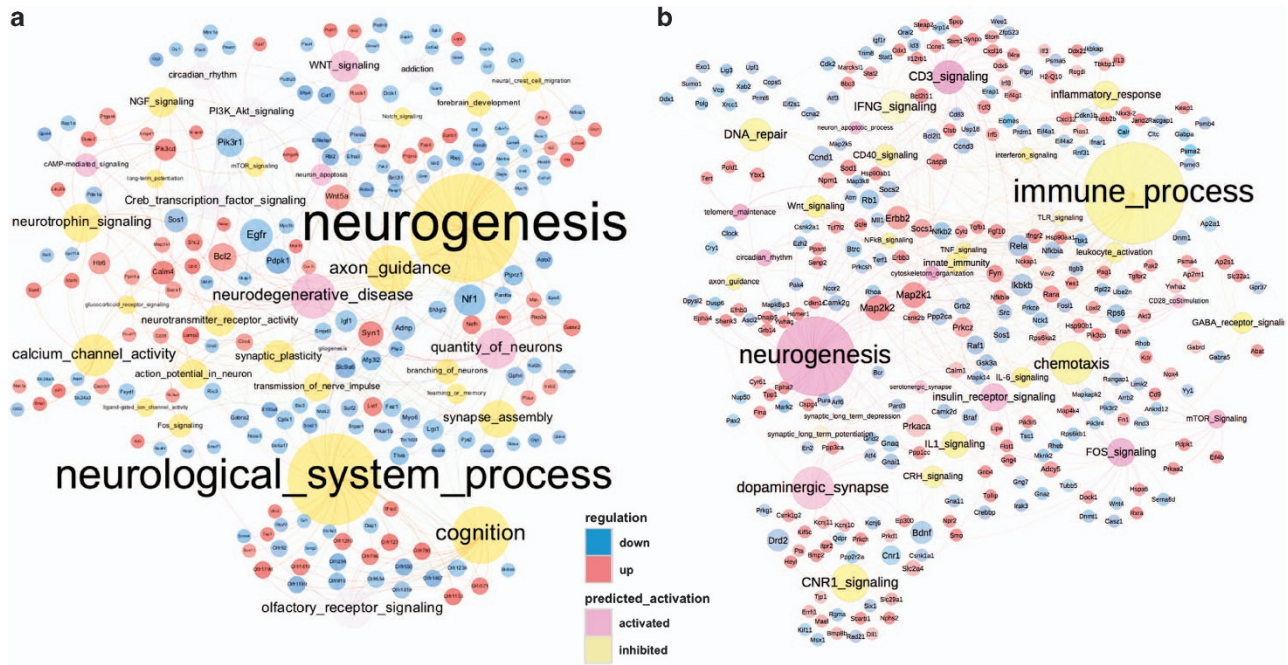

Figure 2. (**a**) Functional network for differentially expressed genes common among blood, spleen and hemibrain that were associated with neurological processes and pathways. Gene node were colored based on the expression values at day 42 after the 10-day aggressor exposure session (T10R42), and pathway nodes were colored based on their predicted activation states at later time points (T5R10 and T10R42). (**b**) Functional network for processes and pathways associated with differentially methylated probes from hemibrain at the promoter region. Gene nodes were colored based on the methylation values of promoter CpG islands of the gene at day 42 after the 10-day aggressor exposure session (T10R42), and pathway nodes were colored based on predicted activation of methylation states of the pathways as inferred from methylation status of promoter regions of genes involved in the respective pathway. Hence, in general, the actual activation of the pathway is expected to be opposite of what is shown here. (These pathways and processes were enriched using hypergeometric test with false discovery rate of $q < 0.05$.)

$P < 0.05$ and FC > 1.5 filter, and were overlapping among blood, hemibrain and spleen (Figure 1a), showed two different temporal trends: 1874 DEGs increased in expression, whereas 1757 DEGs decreased in expression with increasing post-trauma periods (Supplementary Figure 2). These sets of DEGs were mainly associated with immune response (innate immunity and inflammatory responses; Figure 1b) and neurological system processes (mainly neurogenesis; Figure 2a).

At later post-trauma days (T5R10 and T10R42), pathways related to innate immunity and inflammatory responses, such as interferons, Toll-like receptors and tumor necrosis factor-α (TNF-α), and interleukin signaling, chemotaxis and NF-κB-regulated transcriptions were activated. On the contrary, signaling pathways implicated in anti-inflammatory responses (SOCS1, SOCS3, TOB1, CTLA4, IL-1RN and IL-10 signaling) were inhibited (Figure 1b; Supplementary Table 3). Interestingly, however, pathways related to CD3, CD4, CTLA4, TOB1, IDO1 and TGFB signaling were suppressed, suggesting inhibited T-helper cell-type-2 immunity (Figure 1b; Supplementary Table 3), whereas most immune responses were activated. Biological processes related to neurogenesis, synaptic plasticity, learning or memory, mTOR, NOTCH and calcium signaling were inhibited, whereas apoptosis of neurons, WNT- and cyclic AMP signaling was activated at later post-trauma periods (Figure 2a).

Consistent with the above findings, upregulated central nodes in the regulatory networks for the overlapping DEGs (Irf8, Ddx58, Nfκb1, Il6, Irf1, Cebpb, Bcl2 and Trim8) were involved in inflammatory responses (Supplementary Figure 3), whereas downregulated central nodes were involved in immunodeficiency processes (Pik3r1) and modulating synaptic plasticity (Tal1).[19]

Enrichments of significantly ($q < 0.05$) hyper- and hypo-methylated promoter regions in the hemibrains of the longer-period (T10) stress group at the later (T10R42) post-trauma time point (Figure 1c) showed inhibited promoter-methylation patterns of genes related to inflammation and innate immune responses, and increased methylation patterns at promoter regions related to neurogenesis, synaptic plasticity, activities of dopaminergic synapses, circadian rhythm, telomere maintenance, and FOS and mTOR signaling (Figures 1d and 2b). This is consistent with enrichments and activation states of DEGs common among brain, blood and spleen (Figures 1b and 2a).

In summary, at 42 days after the 10 days of trauma (T10R42)—believed to roughly approximate chronic PTSD—functional networks of DEGs common among brain, blood and spleen showed inhibition of neurogenesis, synaptic plasticity and processes related to learning and memory, which was further supported by changes in DNA methylation patterns in the hemibrain. On the other hand, signaling pathways related to inflammatory responses were activated, whereas anti-inflammatory pathways and T-helper cell-type-2 immunity were inhibited.

Additional molecular changes that were associated with PTSD-comorbid pathologies (metabolic disorders, insulin signaling disruption, oxidative stress, dysregulation of mitochondrial and telomere maintenance) were also significantly associated with commonly altered transcripts (Table 1; Supplementary Table 4).

Molecular alterations in brain regions implicated in fear and traumatic stress responses

We further investigated stress-induced molecular alterations in brain regions implicated in traumatic stress response: AY, HC and MPFC. Others have shown that, under traumatic stress, the dentate gyrus of HC (an active-site of neurogenesis in the adult brain) is highly affected by hippocampal inflammation,[20] amygdala activity[21] and MPFC neuronal inputs,[22] making these three

Table 1. Differentially regulated genes (common among blood, hemibrain and spleen) that were significantly associated with signaling pathways implicated in PTSD comorbidities

Signaling pathway	*Differentially expressed genes*
Calcium signaling pathway	RYR2, GNA14, CYSLTR1, ADORA2A, ADRA1B, PTGER1, HRH2, CALML5, ATP2B1, ATP2B2, CD38, ATP2A2, ERBB3,GNAL, GNAS, PDE1C, PDE1A, EGFR, CACNA1S, ADCY1, HTR6, EDNRB, PLCB2, ADCY3, ADCY5, CAMK2B, GRB2
cAMP signaling pathway	RYR2, RAC2, EP300, ADORA2A, CALML5, ATP2B1, ATP2B2, ATP2A2, ADCYAP1R1, PIK3R1, GIPR, GNAS, PDE4D, VAV3, VAV2, CACNA1S, ACOX1, ADCY1, ATP1B1, HTR6, NFKB1, PPP1CC, AKT1, ADCY4, ADCY5, ADCY9, ADORA3, ADRA2A, ADRBK1, AKAP6, CAMK2B, CHRM1, FLNA, GLP2R, GNAL, GRM6, GRM7, OPRD1, OPRM1, PDE1A, PTGER4, RAPGEF4, RGS4, RIMS2, RLF, RPS6KA1, S1PR3, GPR44
Insulin signaling pathway	PIK3CD, ACACA, GSK3B, EIF4EBP1, CALML5, PDPK1, PRKAB2, PRKAB1, GRB2, SHC2, PIK3R1, PTPRF, PPP1R3E, RPS6, SOS1, TSC1, CBLC, PPP1CC, PTPN1, EIF4E2, AKT1, INPP5D, PTPRA, ADIPOR1, CD38, FFAR1, GIPR, GJA1, IRS1, JAK2, LARS, MGEA5, PFKFB2, SERP1, SOCS1, TCF4, TCF7L2, TRH, ADCY1, ADCYAP1R1, ATP1B1, CDK2, CPLX1, CPLX3, CRKL, CSNK2A1, DOK5, DOK7, FGF23, FOXO1, GNAS, IKBKB, IRS4, KIF5B, KLB, LPIN1, MYO5A, NRAS, PTPN2, RAF1, RAPGEF1, RAPGEF4RIMS2, RYR2, SHC4, SMARCC1, SOCS4, STAT1, STXBP4, CALM1, CALM3, CALM4, CRK, FGFR4, IRS3, KRAS, MAP3K14, MAP3K8, MAP4K3, MAP4K5, MAPK6, PDE3A, PIK3C2G, PRKCB, PRKCH, PRKCQ, RHOJ, SNAP23, VAMP2
Mitochondrial biogenesis and translation	MEF2D, HDAC3, TFAM, PPARGC1B, TGS1, PRKAB2, PRKAB1, HELZ2, MAPK12, BAK1, DAP3, GFM2, MRPL23, MRPL24, MRPL33, MRPL44, MRPL49, MRPL9, MRPS15, MRPS17, MRPS23, MRPS25, MRPS33
mTOR signaling	AKT1, CDK2, CLIP1, EIF3D, EIF4A1, EIF4B, EIF4E2, EIF4EBP1, IGF1, PDPK1, PIK3CD, PIK3R1, PPP2R5E, RHOF, RPS3, RPS6, RPS6KA1, RRAGD, TSC1, VEGFA, PRKCB
Oxidative stress response	DUSP10,DUSP3,DUSP7,BCL2,MYC,DDIT3,STAT1,MAPK12,CD24A,TPO,NCF2,CAT,CYCT,CYBB,DNAJB14, DNAJB4,DNAJB6,DNAJB9,DNAJC10,DNAJC11,DNAJC16,DNAJC17,DNAJC5,DNAJC9,SEC63
Telomerase maintenance and aging	TP53, BCL2, MYC, POLR2A, AKT1, SMC6, NSMCE2, NFKB1, PPP2R5E, XRCC6, CABIN1, CDK2, HIRA, HMGA2
Type I and II diabetes mellitus	CD80, FAS, IRF1, H2-AB1, H2-EB1, H2-K1, H2-M10.2, H2-T22, TRAV3N-3, AKT1, PIK3CD, PIK3R1
VEGF signaling pathway	RAC2, ELMO1, BAIAP2, PTK2, CYBB, PDPK1, SHC2, PIK3R1, NCF4, CTNNA1, DOCK1, VAV3, VAV2, CDC42, SHB, SH2D2A, MAPK12, AKT1, CTNND1, GRB2, ITGA1, ITGA4, ITGB1, KRAS, PIK3C2A, PLA2G4A, PRKCB, SOS1, VCAM1, VEGFA, RAF1, PTPN2
WNT signaling pathway	MYH7, MYH6, MYH8, CDH16, CSNK1D, GSK3B, GNA14, EP300, ARRB2, HDAC3, HDAC8, PCDH1, PCDH9, PCDH7, PCDHB5, PCDHB6, PCDHB4, CSNK1A1, WNT6, LEF1, GNG5, CTNNA1, PCDH20, TGFBR1, PCDH10, FRAT1, MYC, GNB1, PCDHB11, PCDHB10, PCDHB16, CTNNAL1, CDH9, FAT3, FZD9, FZD3, FZD6, DKK1, WNT5A, ACVR1C, PLCB2, TCF7L2, WNT2B, RAC2, VRK2, CDC42, CSNK1E, CSNK2A1, CSNK2A2, CSNK2B, CTNND1, DDIT3, FBXW2, HHEX, HOXB9, LDB1, MITF, PIAS4, PYGO2, RNF138, RYR2SENP2, SFRP4, TCF3, TCF4, TGFB1I1, TLE2, TLE3, VANGL2, WNT3, WNT5B, AKT1, CAMK2B, CUL1, G2E3, HECW2, NFATC1, NFATC3, PPP2R5B, PPP2R5E, PPP3CC, PRICKLE1, PRKCB, RAC3, ROCK1, ROCK2, WWP2, DAB2, DLG1, FHL2, HIPK2, RAF1, RUNX2, SUMO1, TRP53

Abbreviation: PTSD, post-traumatic stress disorder.

brain regions noteworthy[23,24] for additional molecular profiling. Differentially regulated transcripts in the AY, HC and MPFC across four time points were significantly associated with inflammatory pathways (TNF-α, IFN-γ, IL-6, chemokine, and TLR signaling and NF-κB transcriptional activities), as well as other important signaling pathways such as histone modifications, oxidative stress, neuronal growth factors, and WNT, NOTCH, β-catenin and cyclic AMP signaling pathways (Figure 3a).

In the AY, signaling pathways of inflammatory responses, neurogenesis and synaptic plasticity were activated earlier (a day after stress) in response to trauma, and inhibited at later post-trauma days (42 days after stress) (Figure 3a; Supplementary Figure 4). Suppressed expression of DEGs in the AY at later post-trauma days (Figure 3a; Supplementary Figure 5a) is likely related to the role of the AY as an early fear-response center.[25] Notably, as reduced basolateral amygdalar activity or lesions suppress adult neurogenesis, DEG suppression in this region may prevent selective activation of newborn neurons[26] and contribute to decreased hippocampal neurogenesis.

In the HC and MPFC, inflammatory signaling pathways were activated at earlier time points (T5R1) and remain activated at later post-trauma days (T10R42), whereas neurogenesis and synaptic plasticity were inhibited at T10R42 (Figure 4a). Particularly, DEGs from HC were associated with signaling pathways of VEGF, IFN-γ, IL-6, TNF-α, NF-κB, Toll-like receptors, chemotaxis, calcium, notch and circadian rhythm (Supplementary Figure 5a). In addition to evidence of activated inflammatory pathways, expression levels of important pro-inflammatory molecules (IFN-γ, IL-6, TNF-α, IL-1B, CRP, CCR2 and others) were increased in the HC and MPFC at earlier and, to a lesser extent, at later post-trauma days (Figure 3b; Supplementary Figures 5b).

Activated inflammatory responses, inhibited neurogenesis and inhibited synaptic plasticity in HC and MPFC are consistent with the activation of inflammatory pathways and inhibition of neurogenesis for overlapping DEGs among the hemibrain, blood and spleen observed at later post-trauma days (Figure 4b). They are also consistent with downregulated neuronal growth factors (BDNF, DLG4, IGF1, VEGFA, ITGAL and EGFR) in overlapping DEGs at later post-trauma days leading to inhibited neurogenesis (Figure 4b).

To further corroborate our molecular indicators of inhibited neurogenesis in Agg-E mice, we used a (Y-maze) spatial learning and recognition memory (novelty cognition) test, which is reported to involve newly generated neurons,[27–29] although the association of this test with neurogenesis is inconsistent across studies.[30] The stressed, Agg-E mice showed poorer performance than the C-ctrl mice ($P < 0.09$) at T10R1 (Figure 4c), suggesting impaired neurogenesis in the dentate gyrus of the HC, which corroborates the molecular findings regarding inhibited neurogenesis.

In summary, we observed activated inflammatory signaling pathways early on (T5R1) in the HC and MPFC, and activation persisted even at 42 days after the 10 days of trauma (T10R42) in HC and MPFC. In both the HC and MPFC, we observed not only the activation of downstream signaling pathways of pro-inflammatory molecules but also increased expression levels of the pro-inflammatory molecules themselves at T5R1 (including those shown to inhibit neurogenesis: TNF-α, CRP, IL-6, IFN-γ, IL-1B and CCR2) as verified using an independent nanoString assay

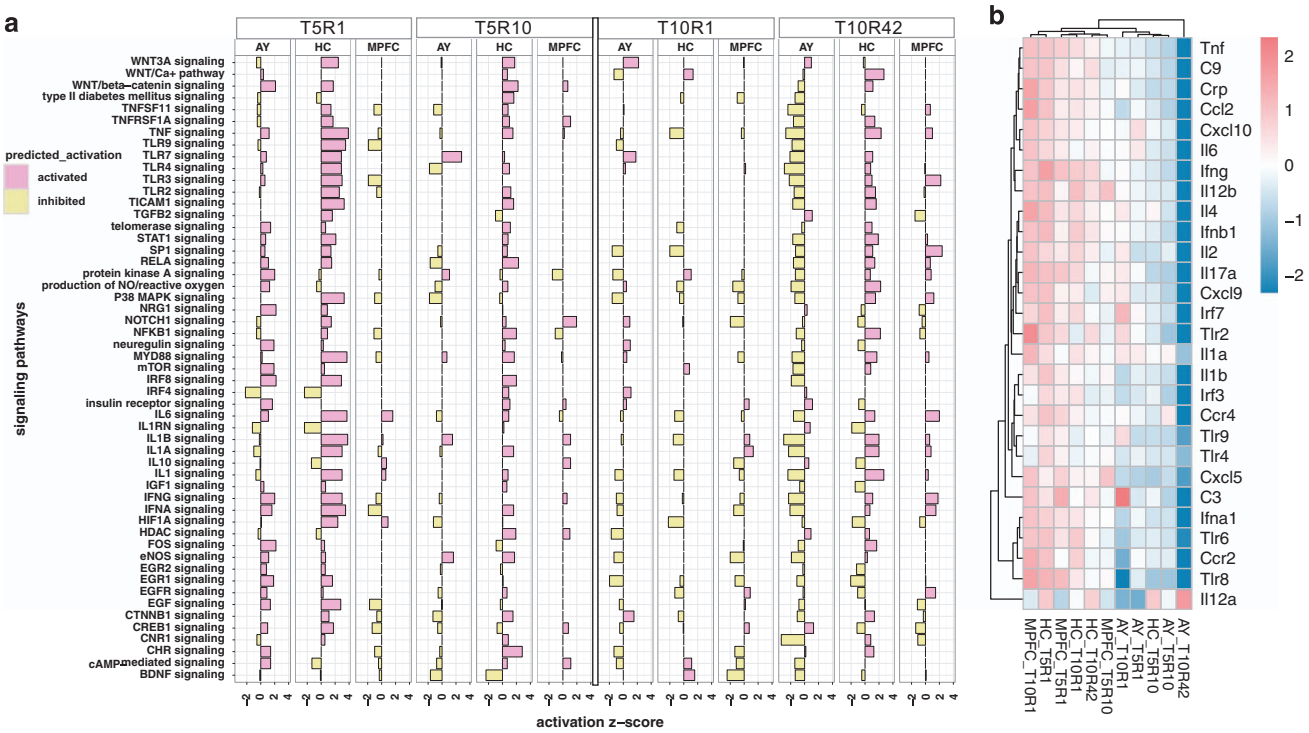

Figure 3. (**a**) Activation states of inflammatory response, WNT signaling, insulin signaling, telomere dysregulation and growth factor-related signaling pathways in amygdala (AY), hippocampus (HC) and medial prefrontal cortex (MPFC) at four different time points of day 1 of after the 5 days of trauma (T5R1) and 10 days of trauma (T10R1), day 10 of after the 5 days of trauma (T5R10), and day 42 of after the 10 days of trauma (T10R42). (**b**) Select pro-inflammatory molecules from ~120 transcripts assayed using nanoString's 255 pro-inflammatory probes. About 120 transcripts (Supplementary Figure 5a) passed fold change > 1.5 and a significance of $P < 0.1$ in AY, HC and MPFC at least at one time point in each brain region.

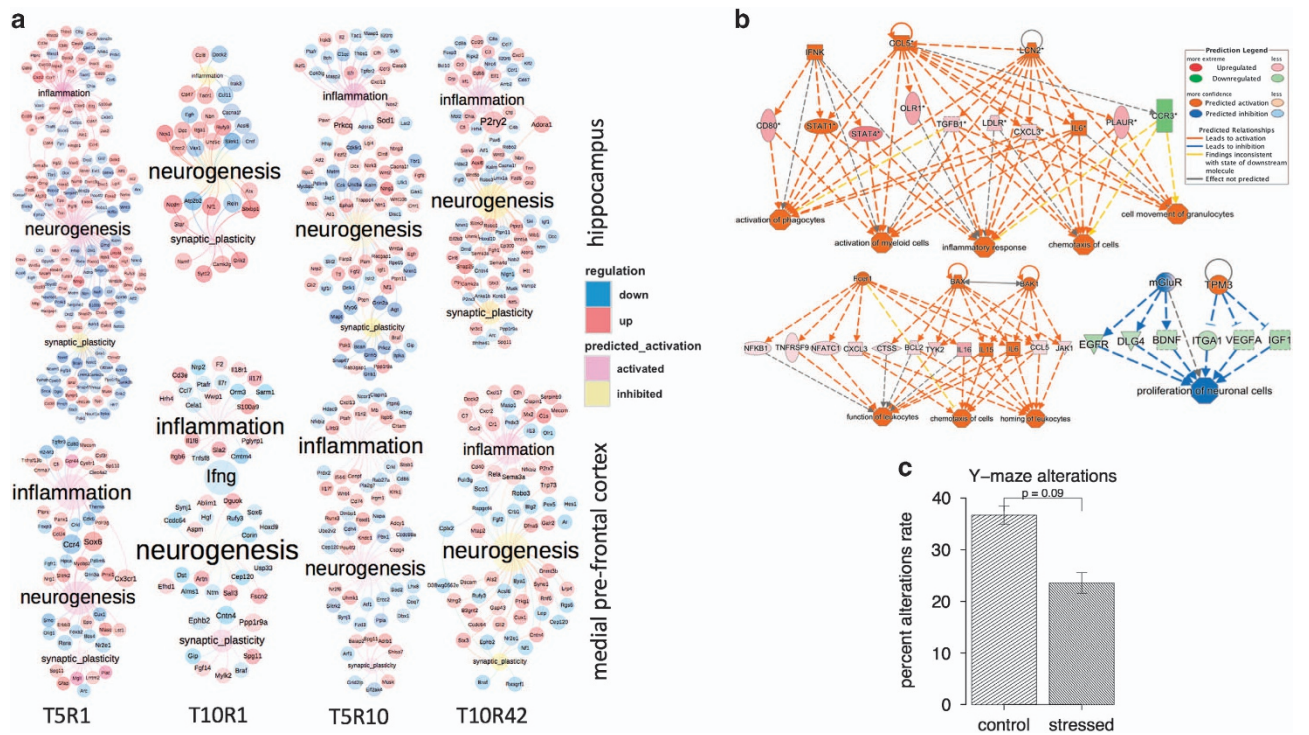

Figure 4. (**a**) Functional networks of differentially regulated genes in hippocampus (top panel) and medial prefrontal cortex (lower panel) at day 1 of after the 5 days of trauma (T5R1) and 10 days of trauma (T10R1), day 10 of after the 5 days of trauma (T5R10), and day 42 of after the 10 days of trauma (T10R42). (**b**) Regulatory networks of differentially expressed genes common among blood, hemibrain and spleen at later post-trauma days (T5R10 and T10R42). (**c**) Y-maze assessment of aggressor-exposed (stressed) and control C57BL6 mice at T10R1.

(Figure 4b). Even at 42 days after the 10 days of traumatic stress, inflammation remained activated in the HC (though to a lesser extent). By contrast to inflammatory responses, pathways involved in neuronal growth factors (neurogenesis) were inhibited. Activated inflammation and inhibited neurogenesis at T10R42 in brain regions implicated in fear memory and extinction (HC and MPFC) again corroborate our findings from overlapping DEGs among hemibrain, blood and spleen.

Blood transcripts versus plasma proteins

We observed concordance between levels of plasma proteins at two time points (T5R1 and T10R1) and expression levels (directions) of corresponding transcripts in the whole blood, despite the different cohorts of mice used for plasma and whole blood (transcriptome) assays. Eight genes (TPO, IL-1B, MPO, MMP9, VEGFA, CRP, TIMP1 and APCS) were consistently upregulated in both acute (T5) and chronic (T10) trauma sessions at the levels of both protein and transcript expression (Supplementary Figure 6a). Within each exposure session, 13 out of a total of 16 genes at T5R1, and 11 out of 16 genes at T10R1 were expressed in the same direction at both the protein and transcript levels in each exposure session (Supplementary Figure 6a). Stress-induced alterations of these proteins, along with dysregulation of immune response pathways, may underlie the maladaptive responses that can potentially progress to specific behavioral disorders and somatic pathologies.

Validation of transcripts from hemibrain and spleen

We also validated DEGs from the hemibrain. Although some of the assayed transcripts were not among significant DEGs, we observed a significant positive correlation mostly in direction and in many cases in magnitude of FCs (Supplementary Figure 6b). For DEGs from the spleen, the nanoString assay showed a directional correlation with microarray findings (Supplementary Figure 6c).

Overall, our mouse model made it feasible for temporal sampling of important brain regions and organs for system-level analysis of molecular events that are otherwise inaccessible from patients. This allowed us to identify differentially expressed transcripts, methylated promoter regions across multiple time points and associated signaling pathways that reveal potential molecular mechanisms for stress-induced disorders. Molecular changes at earlier time points provided molecular signatures for disease onset and progression, whereas those at later time points are indicators of disease persistence.

DISCUSSION

We have identified temporal and overlapping DEGs across multiple tissues, and associated gene interaction networks in a mouse model simulating aspects of PTSD. Such approaches provided comprehensive assessment of the molecular underpinnings of the major aspects of this debilitating disorder. Specially, determining the activation status of significantly altered pathways or processes has made it possible to infer behavioral responses (phenotypic outcomes) of stress-induced molecular alterations.

Our integrative and consensus network analyses of the various blood, brain and spleen data sets suggest that stress-induced inflammation may be negatively affecting brain regions responsible for cognition and traumatic fear memory extinction leading to behavioral,[12,14] and systemic disorders including metabolic imbalance,[31] further dysregulation of immune response, disruption of insulin signaling, dysfunction of mitochondrial and telomeric maintenances, and cardiovascular problems.[32] Our findings suggest that activated inflammation at earlier time points in brain regions implicated in fear memory and extinction (HC and MPFC) might cause inhibited neurogenesis at later time points. This is consistent with studies that show causal relationships between hippocampal inflammation and impaired neurogenesis in the dentate gyrus.[20]

Normally, chemokines, cytokines and the family of NF-κB factors are widely expressed in zones of active neurogenesis, where they are critical for maintaining brain health and function.[33,34] This includes long-term potentiation of hippocampal CA1 region,[35] neurogenesis[34,36] and synaptic plasticity that underlies learning and memory.[37] However, over-secretion of pro-inflammatory cytokines has been associated with neurodegenerative and neuropsychiatric diseases.[38,39] For example, elevated levels of IL-6, TNF-α, IFN-γ and NF-κB underlie inflammation-induced cognitive decline,[40] C-reactive protein-mediated inflammation has been associated with PTSD symptoms,[40] and IL-1β-induced chronic neuroinflammation impairs adult HC neurogenesis[41] disrupting production, distribution and recruitment of new neurons into relevant neural networks.[42] Even mild inflammation in the HC is linked to mood changes, memory deficits, reduced neurogenesis and partial loss of principal neurons,[43] whereas chronic peripheral inflammation has been shown to inhibit neurogenesis.[44] Other comorbidities such as diabetes, which we infer to be triggered by inflammation, have also been implicated in impaired hippocampal neurogenesis.[45] Moreover, increased plasma levels of pro-inflammatory cytokines, upregulated targets of NF-κB/Rel transcription factors[46] and molecular indicators of enhanced inflammation have been linked to PTSD vulnerability,[47] and to comorbid somatic diseases in childhood-abuse[48] and combat-related PTSD.[49]

Here observation of prolonged inflammation and inhibited methylation of promoter regions for pro-inflammatory molecules are also consistent with multiple reports that show a correlation between stress-induced inflammation and chronic pain in PTSD.[40,50–53] Stress-induced chronic inflammation is also implicated in chronic pain,[53–55] which may be responsible for the physical complaints possibly contributing to the impaired social and emotional functions of PTSD patients.[56] Additional long-term effects, most notably epigenetic changes in the adult brain, could further exacerbate alterations to long-term memory and synaptic plasticity, contributing to PTSD-related inhibition of neurogenesis and hence cognitive deficit. These molecular observations were phenotypically corroborated by the impaired Y-maze performance of the stressed mice, a behavioral deficit observed for we believe the first time in an animal model simulating aspects of PTSD.

These transcriptome- and epigenome-based correlative findings (and inferred causal relationships between inflammation and PTSD-associated pathologies) support the notion that stressed-induced inflammation triggers or maintains cascades of comorbidities.[57–59] In short, activated neuroinflammation is implicated in inhibited neurogenesis and synaptic plasticity (cognitive deficits) leading to the development and persistence of the behavioral manifestations of PTSD. Whereas, somatic inflammation seems to be directly involved in tissue damage, triggering and maintaining somatic comorbid pathologies.

The association between PTSD and chronic inflammation suggests that treatment of trauma-induced inflammation may have wide-reaching implications for ameliorating PTSD symptoms. Despite inconsistencies regarding roles of specific pro-inflammatory molecules in PTSD,[60] which might be attributed to psychotropic medications, variations in assay methods or blood collection time of the day,[46] inflammatory pathways may serve as therapeutic targets. Hence, diagnosis and treatment strategies designed to address the burden of PTSD may include addressing the inflammatory problem (immune response imbalances) to alleviate chronic pain, and to reverse tissue damage and cognitive deficits potentially rooted in inflammation.

Identification of overlapping molecular changes in multiple tissues,[61] particularly the presence of common molecular

alterations between blood and brain, might confer confidence in using blood as an accessible brain surrogate sample for clinical translation. Such overlapping molecular events potentially provide a convenient method for patient monitoring and PTSD evaluation, leading to better prognosis, diagnosis and treatment strategies.

Data depositions

Genome-wide data sets reported in this manuscript are deposited at the Gene Expression Omnibus of the National Center for Biotechnology Information.

GSE85495: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE85495
GSE68076: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE68076
GSE45035: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE45035

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DISCLAIMER

Research was conducted in compliance with the Animal Welfare Act, and all other Federal requirements. The views expressed are the authors' and do not constitute endorsement by the US Army

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)