Hippocampal gene expression changes underlying stress sensitization and recovery

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Abstract

Chronic and acute stressors have been linked to changes in hippocampal function and anxiety-like behaviors. Both produce changes in gene expression, but the extent to which these changes endure beyond the end of stress remains poorly understood. As an essential first step to characterize abnormal patterns of gene expression after stress, this study demonstrates how chronic restraint stress (CRS) modulates gene expression in response to a novel stressor in the hippocampus of wild type mice and the extent to which these changes last beyond the end of CRS. Male C57/bl6 mice were subjected to 1) a forced swim test (FST), 2) Corticosterone (Cort) or vehicle injections, 3) CRS for 21 days and then a FST, or 4) allowed to recover 21 days after CRS and subjected to FST. Hippocampal mRNA was extracted and used to generate cDNA libraries for microarray hybridization. Naïve acute stressors (FST and vehicle injection) altered similar sets of genes, but Cort treatment produced a profile that was distinct from both FST and vehicle. Exposure to a novel stress after CRS activated substantially more and different genes than naïve exposure. Most genes increased by CRS were decreased after recovery, but many remained altered and did not return to baseline. Pathway analysis identified significant clusters of differentially expressed genes across conditions, most notably the NfKB pathway. Quantitative RT-PCR validated changes from the microarrays in known stress-induced genes and confirmed alterations in the NfKb pathway genes, Ikbα, RelA and Nfkb1. FST increased anxiety-like behavior in both the naïve and recovery from CRS conditions, but not in mice 24hrs subsequent to their CRS exposure. These findings suggest the effects of naïve stress are distinct from Cort elevation and that a history of stress exposure can permanently alter gene expression patterns in the hippocampus and the behavioral response to a novel stressor. These findings establish a baseline profile of normal recovery and adaptation to stress. Importantly, they will serve as a conceptual basis to facilitate the future study of the cellular and regional basis of gene expression changes as well as genetic risk factors and

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

adverse early life experiences that lead to impaired recovery from stress such as occurs in mood and anxiety disorders.

INTRODUCTION

Stress can both facilitate the onset as well as exacerbate the symptoms of a variety of disorders, such as depression, anxiety, and post-traumatic stress disorder (PTSD) (1, 2). Yet, the majority of individuals exposed to stressful life events exhibit normal resilience and recover from the stressful events without developing a psychiatric disorder. The onset of stress-induced disorders has been hypothesized to result from a loss of resilience, where the brain becomes locked-in to a maladaptive state and cannot return to normal functioning (3). Stress exposure can also sensitize individuals to environmental stimuli, where they experience a heightened response to subsequent stress exposures (4, 5). However, little is known about the transcriptional changes that occur during normal recovery from stress or after sensitization to stress.

The hippocampus is highly sensitive to the effects of stress, which can induce both structural and functional changes at the cellular level (6). These adaptations are regulated through alterations in gene expression, which can occur rapidly after an acute stress and may be transient or can endure beyond the end of stress. Acute stress-induced changes can provide a protective benefit, whereas chronic stress can impair hippocampal function (3).

The effects of stress on the hippocampus are highly context dependent and vary with the duration, intensity, frequency, predictability, and even time of day of the stress (6, 7). Some of these effects are mediated by the binding of glucocorticoids to their receptors (GRs), which function as transcription factors when activated (8–10). Researchers have demonstrated that a history of chronic stress can alter the gene expression response to an acute glucocorticoid (GC) challenge (9) and conversely, blocking GRs can alter the response to a stress (11). These studies suggest that exposure to a novel stressor after a period of chronic stress would induce a different transcriptional response than naïve exposure to the same stressor. While there have been several studies examining expression changes after chronic restraint in mouse hippocampus (12–14), the transcriptional differences underlying altered reactivity to a novel stressor after chronic stress exposure have not been characterized. Further, *in vivo* stress manipulations are likely to produce effects beyond those regulated by GRs alone and this distinction has not been well-characterized.

In this study, microarray technology was used to generate an unbiased, high-throughput transcriptional profile of hippocampal gene expression after acute swim stress, corticosterone (Cort) injection, as well as chronic restraint stress (CRS), recovery from CRS and exposure to a novel, heterotypic stressor. Moreover, assessment of anxiety-like behaviors after recovery followed by novel stress exposure was used to link these changes to translationally relevant measures of mood disorders in mice. These profiles provide new

insight into the transcriptional effects of normal recovery from stress and altered reactivity to a novel stressor after chronic exposure and they are intended to establish a baseline profile of normal recovery and adaptation to stress. These results serve as a conceptual basis that will facilitate the future study of the cellular and regional differences in gene expression changes as well as the effects of genetic risk factors and adverse early life experiences that lead to impaired recovery from stress such as occurs in mood and anxiety disorders.

MATERIALS & METHODS

Animals

Adult male C57/BL6 mice (42d old) were ordered from Charles River Laboratories (Kingston, NY). Animals were group housed (n=4–5) in standard cages (28.5x17x13cm) and allowed to acclimate for 7d before experimentation. Mice were kept on a 12-h light-dark cycle (lights off 1800h) in a temperature-controlled room maintained at 21±2°C. Food and water were available *ad libitum*. The Rockefeller University Institutional Animal Care and Use Committee approved all experimental procedures involving animals.

Stress Paradigm & Tissue Collection

Male mice (7 weeks) were subjected to an acute 6 min Forced Swim Test (FST) in 2L of RT water in a 4L beaker. Mice were removed and dried and allowed to recover for 1hr in the home cage before sacrificing by cervical dislocation. Corticosterone (Cort) was suspended in DMSO and mice were injected with 15 mg/kg Cort or vehicle 1 hr prior to sacrifice. CRS began at 7 weeks of age and was carried out in 50mL conical tubes for 2hr/d starting at 1000h for 21 consecutive days. Control mice were left undisturbed. On day 22 mice were subjected to one of three conditions, they were sacrificed, subjected to FST, or allowed to recover in their home cages for an additional 21 days (Supp Fig. 1A). On day 43 the mice allowed to recover were either sacrificed or subjected to FST and sacrificed 1hr later. All groups had unstressed, age-matched controls that were sacrificed concurrently. Separate cohorts were processed for gene expression and behavior to exclude the possibility that behavioral testing altered gene expression. Mice were immediately decapitated, brains were removed and hippocampus was fresh dissected and flash frozen on dry ice before storing at −80°C.

Microarray

RNA was extracted from whole hippocampal tissue using the Qiagen RNeasy Lipid Tissue Mini Kit (Qiagen#74804) with QIAcube (Qiagen 9001292) and on-column DNase digestion. RNA was quantified using a Nanodrop 2000C (Thermo-scientific) and assessed for integrity on a Bioanalyzer (Aglient). cDNA libraries were generated using Applied Biosystems High Capacity cDNA Reverse Transcription kit for hybridization to Illumina MouseRef8-v2 arrays (San Diego, CA) that contain 24,854 annotated transcripts and can accommodate 8 samples per chip. Each mouse was run as an individual to preserve the within group variability (n=4 mice/group, 2 groups/chip, total of 5 chips).

Data was normalized and subjected to statistical analysis using Genespring software (Agilent Technologies, Santa Clara, CA). Probes that passed quality control were filtered for

expression prior to statistical tests (18,948 probes). Pairwise comparisons between conditions were performed with Student's T-Test, and an ANOVA comparing all stress conditions against controls was calculated, both with significance levels at p<0.05. Bonferroni and Benjamin-Hochberg corrections for multiple comparisons were applied, but yielded no genes that reached significance in any comparisons. Significant genes identified by ANOVA were subjected to pathway analysis to identify gene sets representing specific biological process or functions. Genespring incorporates data from publically curated databases, including Gene Ontology (GO) and WikiPathways, for its enrichment analysis.

qRT-PCR

Remaining cDNA was assayed using Taqman Primers (Life Technologies). Samples were run in triplicate in 96 well plates using an Applied Biosystems 7900HT Real-Time PCR Thermocycler. Data was analyzed using the 2 method and were normalized to *Gapdh* as a reference gene (15).

Behavior

Animals for behavioral assays were moved to the testing room 30min prior to the trial for habituation. 1d following the end of stress animals were placed in the corner of an open field (OF) (65x65cm) and allowed to freely explore for 6min. All trials took place between 1000–1400h and were counterbalanced across conditions throughout testing. Behavioral analysis was done using Noldus Ethovision. The following day animals were placed in a closed arm facing the center of an elevated plus maze (EPM) and allowed to explore for 6min each.

RESULTS

Transcriptional profiles are highly distinct between acute stress, Cort injection, CRS, and recovery

In mice subjected to naïve FST, 1,298 genes (39.3% increased; 60.6% decreased) were identified as significant by pairwise comparison of normalized expression levels with age-matched controls using Student's T-test (p<0.05, Fig. 1A). Only 773 genes (42.3% increased; 57.7% decreased) were identified as significant after 21d CRS and 1,101 genes (43.0% increased; 57.0% decreased) were significant when comparing Cort with vehicle injected mice. 3,999 genes (28.1% increased; 71.9% decreased) were significant when comparing the heterotypic stress condition (CRS+FST) with non-stressed controls. There were only 77 genes changed by both naïve FST and CRS, representing only 5.9% and 9.9% of genes, respectively in each group, demonstrating that acute and chronic stress result in distinct changes in gene expression (Fig. 1B). Similarly, only 96 genes were identified in both the naïve FST and Cort injected group, demonstrating that elevation of Cort is not equivalent to the *in vivo* stress response (Fig. 1B). As expected, both vehicle and Cort injections compared with unstressed controls produced an expression profile similar to naïve FST (74.8% overlap), but one that was still distinct from Cort injected (Supp Fig. 2). After recovery from CRS, 689 genes (53.0% increased; 47.0% decreased) were significantly different. Among mice subjected to FST after recovery from CRS, 1,251 genes (54.1% increased; 45.9% decreased) were significant when compared with age-matched controls (Fig. 1A).

Comparison of naïve FST with CRS+FST and CRS+Recovery+FST demonstrates a core of 95 genes that were altered by an acute stress regardless of stress history (Fig. 1C). Many of these genes have been previously identified in the acute stress response and are associated with recent neural activity, including cfos, Arc, and serum glucocorticoid kinase 1 (Sgk1) (Table 1; Supp Fig. 2). The substantially larger number of genes activated by the heterotypic stress condition (CRS+FST, orange circle, Fig. 1C) compared with naïve FST and CRS +Recovery+FST illustrates that chronic stress creates a unique state of transcriptional reactivity to a novel stressor. Among the 327 genes overlapping in the CRS+FST and CRS +Recovery+FST conditions, many have been associated with changes due to chronic stress, such as BDNF (Table 1). Exposure to a novel stress after recovery from CRS demonstrated a largely unique set of genes from either the naïve FST or CRS+FST conditions (Green circle, Fig. 1C), suggesting that recovery reduced the transcriptional response to a novel stressor, but was still distinct from the naïve response.

A scatter plot of normalized intensity values for all probes passing quality control comparing CRS with RecCRS illustrates that the majority of genes fall into the lower right quadrant (10,682 probes), indicating that they are increased by CRS, but then decreased after recovery (Fig. 1D). Conversely, 3,618 probes are decreased by CRS, but increased in recovery. The upper right and lower left quadrants reflect genes whose direction of change is consistent across conditions, where 2,905 probes were increased and remain increased and 3,608 probes are decreased and remain decreased. Probes identified as significantly different from unstressed controls after CRS (blue, 776 genes) and Recovery+CRS (red, 691 genes) are highlighted and select examples are presented from each quadrant. Genes such as the glutamate transporter EAAT2 (Slc1a2, lower left quadrant) are decreased after CRS and remain significantly decreased after recovery from CRS relative to unstressed controls, whereas genes such as TNFα receptor associated factor 4 (Traf4, upper left quadrant) are significantly decreased after CRS, but elevated after recovery from CRS. A comparison of these gene lists identified only 36 genes that overlap (Fig. 1E, Table 1), indicating they were significantly altered by CRS and remained different from unstressed controls even after recovery. The minimal overlap between these groups also indicates that there are novel transcriptional changes occurring during the recovery process, distinct from those genes that return to baseline.

Pathway analysis

An ANOVA across all stress conditions identified 8,269 genes reaching statistical significance ($p < 0.05$). There was substantial overlap between the gene lists generated from the ANOVA and earlier pairwise comparisons, with over 70% of the same genes identified in the naïve FST and 66% with the CRS condition. Pathway analysis of genes significant by ANOVA identified functionally related groups of genes based on the number altered within known annotated pathways (Table 2). The TNFα-Nfκb signaling pathway was highly significant with 89 of the 184 genes in the pathway significant by ANOVA. Consistent with stress-induced activation of inflammatory pathways, other significant pathways included the B-cell receptor, T-cell receptor and IL-6 signaling pathways. Several genes were implicated in multiple pathways, such as EGFR, Gsk3β, and RelA, suggesting they may serve as nodes of signal integration after stress. Insulin signaling was a highly significant pathway,

implicating metabolic changes after stress as important in the hippocampus and supporting previous evidence that insulin resistance can impair cognitive function(16). Lastly, 173 genes involved in mRNA processing were identified as significant and likely play a role in the changes in gene expression.

Validation of Microarray results

Genes predicted by the literature to be changed by stress that were identified as significant on the microarrays were validated using qRT-PCR. cFos is an early response gene that has been shown to be rapidly activated in response to stress and is a marker of recent neural activity (17) that was significantly elevated in all the FST conditions by microarray (Table 1). cFos was significantly increased in naïve FST and CRS+FST by qRT-PCR (t(6)=5.04, p<0.01, t(6)=3.10, p<0.05), and was increased but did not reach significance (p=0.11) in the CRS+Rec+FST condition (Fig. 2A). Brain derived neurotrophic factor (BDNF) has been shown by numerous groups to be highly dynamic in the hippocampus in response to stress (18). Microarrays identified BDNF as significantly increased in the FST animals that had a stress history and trended toward an increase in naïve FST animals (Table 1). qRT-PCR showed a trend toward an increase of BDNF in the naïve stress and a significant increase after CRS (t(6)=3.32, p<0.05), but no change in the CRS+FST condition (Fig. 2B). After recovery from CRS, basal levels of BDNF remained significantly elevated compared to non-stress controls (t(6)=3.37, p<0.05), and exposure to a novel stress suppressed the elevated BDNF levels back below baseline (t(6)=2.79, p<0.05). Finally, the glucocorticoid receptor (GR) gene (*Nr3c1*), an essential transcription factor mediating stress-induced changes in the hippocampus (19), was not altered by any of the acute stress conditions, but trended toward a decrease after CRS on the microarrays. qRT-PCR confirmed the decrease after CRS as significant (t(14)=3.516, p<0.01), but also found it to be further reduced by heterotypic stress (CRS+FST)(Fig. 2C, t(10)=6.59, p<0.0001). *Nr3c1* levels were found to be significantly elevated in CRS+Recovery (t(6)=3.02, p<0.05), but were not altered by exposure to an acute heterotypic stressor. Together, these data suggest that the microarrays were accurate in identifying genes altered by stress and, moreover, they revealed genes of interest, some of which respond differently in naïve, CRS and recovered CRS conditions.

Changes in Nfkb-dependent gene transcription

qRT-PCR analysis of three central Nfκb family members, *nfkbia*, *rela*, *nfkb1*, was carried out to validate changes identified on the microarrays and to characterize the pathways role in stress-induced changes in gene transcription (Fig. 2). *Nfkbia* was increased in all 3 FST conditions on the microarrays (Table 1), and by qRT-PCR a significant elevation was identified in the naïve FST (t(6)=5.11, p<0.01), and in CRS+FST (t(3)=5.841, p<0.05), but no difference was observed in CRS+Rec+FST where within group variability was high. *Rela* was identified as significant by ANOVA, and by qRT-PCR was significantly increased at the end of CRS (t(6)=14.87, p<0.0001), but not in naïve FST, and levels returned to normal after recovery from CRS. Finally, while *Nfκb1* did not reach significance on the microarray, qRT-PCR analysis showed that it was significantly elevated after naïve FST (t(6)=2.72, p<0.05) and CRS (t(6)=10.23, p<0.0001), and the direction of these changes was consistent with the trends observed on the microarray. Similar to BDNF, the direction of the response to an acute stress was changed for *Nfκb1* after exposure to a chronic stress. Yet, unlike

BDNF the levels of *Nfκb1* returned to baseline after recovery from CRS, and a heterotypic stressor did not induce any significant response.

Anxiety-like behaviors after a novel stress are altered by prior CRS

Open field (OF) and elevated plus maze (EPM) were used to assay anxiety-like behaviors after each stress condition. Exposure of a naïve animal to FST on the preceding day significantly increased the latency to explore the center of the OF suggesting increased anxiety (Fig. 3A, t(31)=3.68, p<0.001). Interestingly, FST on the day after the end of CRS failed to alter the latency to center. Recovery from CRS and then exposure to FST significantly increased the center entry latency (t(31)=2.72, p<0.05). Together, these data suggest that exposure to a naïve acute stress (FST) can increase anxiety-like behavior in OF, but exposure to that same stressor one day after a prolonged stress-exposure (CRS) fails to increase anxiety. However, after recovery from CRS, exposure to a novel stressor is once again able to induce anxiety-like behaviors. Latency to enter the open arms of the EPM was not significantly altered across any conditions except CRS+Recovery+FST, suggesting that a novel stress event after a history of stress can increase anxiety-like behaviors as measured by the EPM (Fig. 3B).

DISCUSSION

This study reveals highly distinct gene expression profiles in the hippocampus that are mediated by the stress history of the subject and thus open the way to investigation of gene expression in animals that are vulnerable to mood related disorders because of genetic or developmental influences. Chronic stress can sensitize individuals to novel stressors (11, 20). Here, the genes underlying stress sensitization are revealed by the vastly different expression profile observed in the heterotypic stress condition (CRS+FST) compared with naïve FST exposure, and this effect lasts beyond the end of stress (Fig. 1C). While the majority of genes returned to baseline in recovery, several thousand genes remain either elevated or decreased for 3 weeks after stress has ended (Fig. 1D). Cort challenged mice showed an elevation of predicted genes such as Sgk1, but also a response that was distinct from naïve FST, indicating that many of the stress-induced changes are not solely driven by activation of the GR or MR (Fig. 1B). Assays of anxiety-like behaviors suggest that naïve FST can induce anxiety, but a novel stress immediately after CRS fails to induce anxiety (Fig. 3). Yet, after recovery from CRS, the stressor can again induce heightened anxiety. Despite the behavioral similarly between naïve and recovery conditions, gene expression profiles remained highly distinct, demonstrating that stress history permanently alters future stress reactivity. These profiles reveal gene expression changes that are long lasting as well as gene expression patterns essential for normal recovery, and they establish a baseline for comparison with genetic mutations that impair recovery from stress and can lead to the development of a mood disorder.

Several reports have used microarrays to study stress-induced changes in the rodent hippocampus (21–23), but to our knowledge this is the first to examine changes induced by a novel stress exposure following recovery or immediately at the end of chronic stress. Differences identified by naïve FST are consistent with results from previous studies of

acute stress, identifying changes in cfos, Arc, and Sgk1 (24–26). Similarly, many of the genes altered by CRS, such as BDNF and GR were also found by others (27, 28), suggesting that this stress paradigm reflects findings of previous work and provides a valid baseline for comparison of genes changed after recovery or exposure to a novel stress.

Changes in BDNF transcript levels are one of the most frequently examined end-points after any stress paradigm, with initial studies showing that it is increased by acute stress (29), but decreased by CRS when assayed immediately after the final stress session (27). However, recent work has shown that BDNF levels remain highly dynamic. The current data and previous reports of BDNF level taken 24 hrs after the final CRS session have found that the basal levels are actually shifted higher (30). However, exposure to another restraint session induces a reduction in BDNF transcripts, and not the increase observed after a naïve stress (31). Our data using a novel stressor after CRS supports this interpretation (Fig. 2B) and expands on it to show that the elevated baseline levels of BDNF remain higher after recovery (Fig. 2C), illustrating a possible mechanism to facilitate recovery from chronic stress in normal mice. We hypothesize that future studies of mice with impaired resilience, either through genetic or environmental manipulations, may not show this continued elevation of BDNF after recovery from chronic stress. Interestingly, exposure to a novel stressor still decreased BDNF levels after recovery (Fig. 2C), which is opposite from the naïve response. The discovery that this effect persists beyond the end of chronic stress illustrates another possible mechanism underlying lasting sensitization to novel stressors.

The stress response and inflammation are closely linked, as GCs can inhibit inflammation through several mechanisms involving GR and Nfkb1(32). Further, abnormal inflammatory cytokine signaling has been linked to major depression and may play a role in the development of psychiatric disorders after stress (33, 34). In the hippocampus, the Nfkb pathway is up-regulated during chronic stress (35, 36), and stress-dependent decreases in hippocampal neurogenesis can be blocked by Nfkb inhibitors (37). Recent work has shown that co-activation of the GR and Nfkb pathway *in vitro* alters the binding site repertoires and gene expression profiles of each (38). The elevation of Cort (Supp. Fig. 3) and increased levels of Nfkbia suggest a coactivation of GR and p65/p50 during naïve FST.

Group-housed rodents habituate to CRS and no longer exhibit an elevation of Cort during restraint sessions (24, 39, 40), and yet RelA and Nfkb1 remained elevated at the end of CRS, when Cort levels are not elevated, suggesting preferential activation of gene targets in the NfkB pathway at the end of CRS. Exposure to the novel stressor (FST) at the end of CRS significantly elevated Cort levels (Supp. Fig. 3) at a time when RelA and Nfkb1 levels are already elevated by CRS (Fig. 2), creating a signaling state that is distinct from the naïve stress response. The substantial difference in the gene expression profiles between naïve FST and CRS+FST provide an *in vivo* demonstration of how differential co-activation states of GR and Nfkb1 can alter their gene targets.

Furthermore, it remains unknown the extent to which NfKb and other stress-induced pathways remain activated after the end of stress. In contrast with the lasting changes in BDNF, the differences in Nfkb1, RelA and Nfkbia were no longer evident after recovery. This suggests that their up-regulation was unique to the end of CRS, and therefore plays a

central role in the altered gene expression observed in response to a novel stressor immediately after CRS, but not after recovery. Additionally, the persistence of the changes in BDNF levels may explain why such a different response to FST was still observed after recovery from CRS. Future work will be necessary to determine the mechanisms underlying the gene expression changes that persist after recovery from CRS and whether such responses differ in animals made vulnerable to stressor via genetic difference or early life stress. Epigenetic regulation is likely to play a role, where changes have been identified in clinical populations (41), and in animal models CRS can induce significant changes in the methylation state of histones in the hippocampus (42, 43). The microarrays in the present study identified changes in numerous genes implicated in epigenetic regulation, such as HDAC 4,5,7–9 and 11, dmnt3l, ep300 and CREBbp (Supp. Table 6), which are likely to play a role in the altered expression profile.

The hippocampus is a functionally diverse brain structure and future studies will seek to characterize the expression profiles from distinct hippocampal subregions and cell types. While the present study is limited by not addressing the subregion or cell type specificity of the changes observed, these findings can serve as a benchmark for comparison with expression profiles from microdissected tissue (9, 10) or studies using cell-type specific genetic reporters to isolate RNA (44). This data is consistent with previous studies such as by Datson et al (2013), where 13 of the 21 genes identified as responsive to Cort or stress in the rat dentate gyrus were also identified here as significant by ANOVA in whole mouse hippocampus, demonstrating that this data set will serve as an important resource for future meta-analyses across species, subregion, and cell-type.

CRS can induce anxiety and depression-like behaviors in mice, although the literature is mixed (45). Increased anxiety-like behaviors appear to persist after recovery from CRS in the rat (46), but little is known about the mouse. The present data suggests that CRS may immediately protect C57/bl6 mice from anxiety induced by a novel stress, as animals in the CRS+FST group did not show any difference from unstressed controls one day later, whereas naïve mice subjected to FST showed a substantial increase in anxiety in the OF (Fig. 3). After the recovery period, this apparent "protective" refractory effect appears to dissipate because exposure to a novel stress at the end of recovery again induced increased anxiety.

Together, these data show that naïve stress, Cort, and chronic stress can induce distinct changes in gene expression and that CRS alters the expression response to a novel stress, potentially through unique targeting of GR and NfKb transcription factors throughout the genome. It is important to emphasize that gene expression changes in recovery are not a return to baseline, despite the normalization of anxiety-like behaviors in response to an acute stress. Future work will be necessary to identify the specific molecular mechanisms (possibly epigenetic) regulating these changes. This work establishes a baseline profile of normal recovery and adaptation to stress that will facilitate the future study of impaired recovery from stress that occurs in mood disorders. Comparisons with genetic and environmental manipulations proven to increase anxiety-like behaviors, such as early life stress, will identify genes that fail to recover in models of increased susceptibility to mood disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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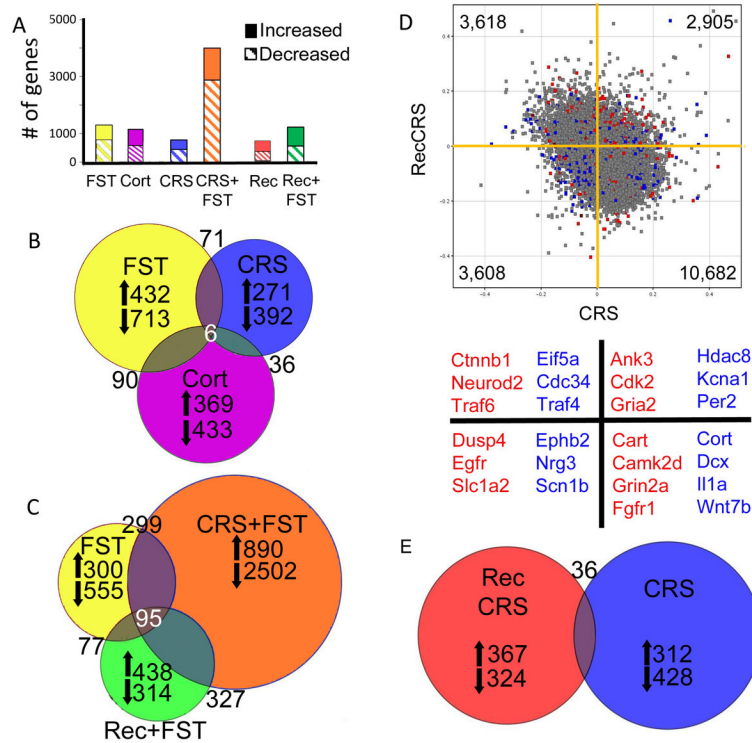
Figure 1. Gene expression changes in hippocampus in response to stress depend on the prior stress history of the subject

(A) Solid bars represents the number of significantly increased genes and hatched bars represent significantly decreased genes identified by pairwise comparisons of each stress group with age-matched controls (t-test, $p<0.05$, n=4 mice/group) (Yellow = naïve FST, purple = Cort vs. vehicle injected, blue=CRS, orange=CRS+FST, red=recovery from CRS, green=recovery from CRS+FST). (B) Proportional venn diagram illustrating the genes significantly altered by both the acute stress (FST=yellow), chronic stress (CRS=blue), and Cort injection (Cort=purple) conditions. The numbers of genes unique to each comparison that were increased or decreased are listed next to arrows indicating the direction of change. (C) Venn diagram of genes altered by each FST condition reveals a core of 95 genes that were always changed by this stressor. The large numbers of unique gene expression changes in each condition shows that the response to FST is altered by the stress history of the group (yellow=naïve, orange=CRS+FST, green= Recovery from CRS+FST), with the vast majority of changes occurring when the animal is exposed to a novel stressor immediately after a chronic stress exposure (orange circle). (D) Scatter plot of normalized expression values for each microarray probe comparing CRS (x-axis) with recovery from CRS (y-axis). The majority of genes are increased by CRS, but decreased after recovery (10,682, lower right quadrant), however, there are a number of probes that are increased by CRS that remain elevated after recovery (2,905, top right quadrant) or are suppressed by CRS and remain low in recovery (3,608 probes, lower left quadrant). Highlighted probes are those that reached significance when compared with age-matched controls (blue=CRS, red=recovery from CRS, gray=not significant). Several examples of the highlighted genes are listed below the scatter plot by color designation and quadrant. For example, blue points

in the lower left quadrant, such as Nrg3 and Scn1b, represent genes that are significantly changed by CRS when compared with unstressed control and are also decreased after recovery from CRS. Whereas red points in the upper right quadrant, such as Cdk2 and Gria2, are genes that remained significantly different from controls after recovery from CRS, and were also increased immediately following CRS. (E) Venn diagram illustrating that the number of genes significantly different from controls after recovery from CRS (red) are mostly unique from those significantly altered by CRS (blue).
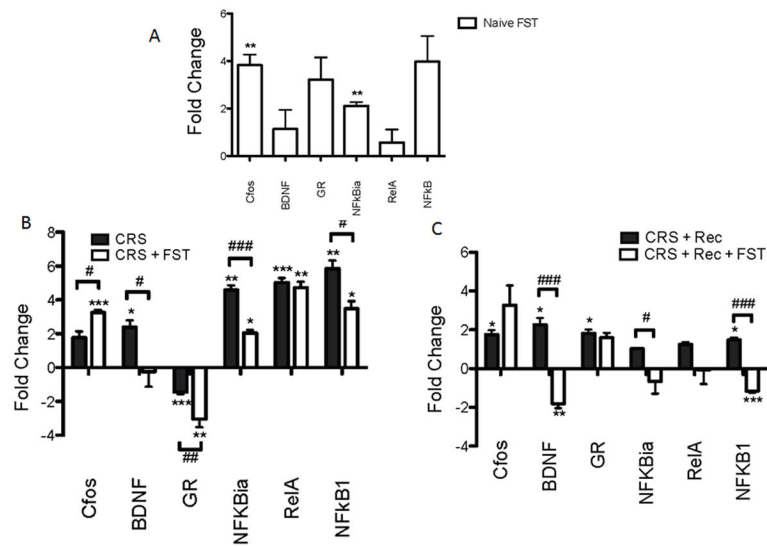
Figure 2. qRT-PCR validates differences in genes identified by microarray known to be associated with the stress response and NfKb signaling

Bar graphs representing fold change from controls on genes measured by qRT-PCR. (A) Naïve FST significantly increased mRNA levels of cfos and nfkbia (**$p<0.01$ v. control). BDNF, GR, RelA and Nfkb1 all trended toward an increase but did not reach significance by qRT-PCR. (B) CRS (black bars) and CRS+FST (open bars) resulted in significant increases or decreases in several genes. Of note, BDNF is elevated by CRS, but decreased compared to control in CRS+FST (#$p<0.05$ between stress groups). The CRS levels of all Nfkb pathway members is elevated by CRS (Nfkbia **$p<0.01$, Rela ***$p<0.001$, Nfkb1 **$p<0.01$), but the addition of FST significantly decreased levels of nfkbia and nfkb1 (###$p<0.001$ and #$p<0.05$ respectively). (C) Significant genes in recovery from CRS (black bars) and Recovery from CRS + FST (open bars). BDNF showed a similar response pattern as the end of CRS (###$p<0.001$), however, Nfkbia and RelA were no longer significantly different from controls after recovery or novel FST. (*significantly different from control, #significantly different from heterotypic stress)
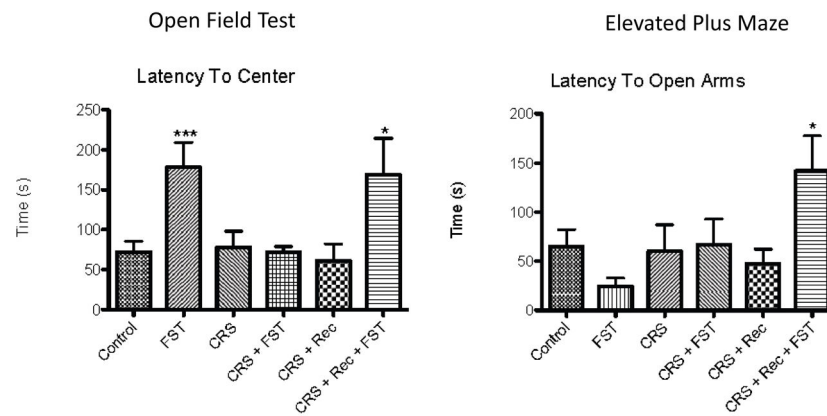
Figure 3. Chronic stress alters anxiety-related behaviors after novel stress exposure and recovery

Latency to enter the center of the open field increased in naïve FST and FST after recovery from CRS, but not by FST immediately after CRS (A). Latency to enter the open arms of the EPM was significantly increased by a novel stress only after recovery from CRS (B). (*$p<0.05$, ***$p<0.001$, n=14 controls; n=10/stress condition)

Table 1

Lists of selected genes identified as statistically significant in overlapping sets

Gene lists are sorted by those consistently increased, consistently decreased, or exhibiting an inverse response across groups. The left arrow indicates the direction of the response to initial condition and right arrow gives the direction of the response in the comparison condition. For example, Cdk2 is decreased after CRS, but increased after recovery when compared with unstressed controls. Representative genes from the naïve FST and CRS overlap of Fig. 1B (column 1), naïve FST and CORT overlap of Fig 1B (column 2), all FST condition overlap in the center of Fig. 1C (column 3), the CRS+FST and CRS+Rec+FST overlap of Fig. 1C (column 4), and CRS and CRS+Rec overlap of Fig. 1E (column 5).

	Genes altered by Acute and Chronic Stress (71 Overlap, Fig. 1B)	Genes altered by Acute Stress and Cort treatment (90 Overlap, Fig. 1B)	Genes always altered by FST (Center 95, Fig. 1C)	Genes altered by FST after chronic stress and recovery from CRS (327 Overlap, Fig. 1C)	Genes altered by CRS and Recovery from CRS (Center 36, Fig. 1E)
Increased	⇑ Axud1 ⇑ Calu (Ca^{2+} bind. prot.) ⇑ Cdkn1a	⇑ Alg14 ⇑ Btd ⇑ CCdc137 ⇑ Commd6 ⇑ Slc25a38	↑ Arc ↑ C-fos ↑ Egr1 ↑ Egr4 ↑ Junb ↑ Nfkbia ↑ Per1 ↑ Per2 ↑ Sgk1	⇑ BDNF ⇑ Myh9 (myosin, heavy chain 9) ⇑ RhoB	⇑ DUSP6 ⇑ Irs2
Decreased	⇓ Atp2b2 (Ca^{2+} transp.) ⇓ Cc2d2a (Ca^{2+} sig.) ⇓ Ephb2 ⇓ Fadd ⇓ Wnt2	⇓ Arfgap1 ⇓ Fkbp7 ⇓ Hdac7 ⇓ Zfp90	↓ Foxq1 ↓ Smarcal1 ↓ AcSF3 ↓ Egfl7 ↓ Hes5 ↓ Megf9 ↓ Hhex	⇓ Cdkn1b ⇓ Cnnm1 (Cyclin m1) ⇓ Cnr1 ⇓ Crebl2 ⇓ Csnk1g1 (Casein Kinase 1 γ1) ⇓ Gria 3 (AMPA) ⇓ Pgr (progesterone receptor) ⇓ RapGEF4 ⇓ RasGEF1c ⇓ Rock2 ⇓ Scn1a (Na$^+$ Channel)	⇓ Pfn2 ⇓ Rasgrp3
Inverse Response	↑⇓ Epha10 ↓↑ Tsn	↑⇓ Ddit4 ↓↑ Egfl7 ↑⇓ Map3K6	N/A	↑⇓ Cdk5r1 ↓↑ Creb3l4 ↑⇓ Pftk1 (Cyclin-dependent kinase 14)	↓↑ Cdk2 ↓↑ Cort ↓↑ Eif5a ↑↓ Shh ↓↑ Zfp13

Table 2

Significant pathways compiled from genes identified as significant by ANOVA

The 8,269 genes identified as significant by an ANOVA across stress conditions were compared against annotated interaction pathways to identify groups of genes known to be functionally linked. Pathways are ranked in order of significance by p-value. The number of significant genes in each pathway (Matched Entities) is listed next to the total number of genes in the pathway (Pathway Entities). Representative genes identified as significant in each pathway are listed in the far right column (Selected Matched Genes).

Pathway	p-Value	Matched Entities	Pathway Entities of Experiment	Selected Matched Genes
Mm_TNF-alpha_NF-kB Signaling Pathway WP246_41391	1.39E-09	89	184	Akt2, Bcl3, BCL7A, Crebbp, Casp2,7, CSNK2A1,2, Eif4a3, Gsk3b, Hsp90aa1, Hsp90ab1, Map2k5, Map3k1,7ip2,7ip2, Nfkbia, e,z, Nkiras1,2, Rel, Rela, b, Smarcc1, Tnfrsf11a
Mm_B_Cell_Receptor Signaling Pathway WP274_41374	2.67E-08	76	156	Bax, Bacl2, Bcl6, Casp7, Ccnd2,3, Cdk2, ctnnb1, Gsk3b, Hdac5,7, Map2k1, Mapk1, Nfatc1,2,3, Nfkbia, Rel, Rela
Mm_mRNA_processing WP310_41325	2.88E-07	173	551	Cdk9, Dicer1, Dnd1, Eif3s4, Eif4e
Mm_Insulin_Signaling WP65_41286	9.96E-06	70	159	Akt2, Egr1, Eif4e, Eif4ebp1, Fos, Gsk3b, Igfr1, Map2k1,3,4,5,6,7, Map3k1,6,9,12, Mapk1,9,19,12,13, Pten, Rapgef1, Rhoj, Sgk, Slc2a1,4
Mm_T_Cell_Receptor Signaling Pathway WP480_41339	1.11E-05	60	133	Abl1, Crebbp, Ctnnb1, Dbnl, Fos, Hdac7, Jak3, Map2k1, Map3k1, Mapk1, Rapgef1, Stat1,5a
Mm_IL-6 signaling Pathway WP387_41281	1.31E-05	48	99	Cdk5r1, Cdk9, Crebbp, Eif2a, Eif4e, Ep300, Fgr, Fos, Gsk3b, Hras1, Hsp90aa1, Il6, Il6ra, Jak1,2, Map2k1,4,6, Mapk1, Sgk, Stat1,3,5a
Mm_EGFR1_Signaling Pathway WP572_41396	2.15E-05	77	176	Camk2a, Egfr, Fos, Jak1,2, Map2k1,3,5,7, Map3k1, Mapk1, Stat1,3,5a, Smad2
Mm_Cytoplasmic Ribosomal Proteins WP163_41303	2.16E-05	33	80	Rpl, Rps
Mm_MicroRNAs_in cardiomyocyte hypertrophy WP156041377	2.25E-05	44	104	Akt2, Camk2d, Ctnnb1, Eif2b5, Fgfr2, Hdac5,7,9, Igf1,1r, Lrp6, Map2k1,3,4,5,6,7, Map3k7ip1, Mapk1, Mtor, Rock1,2, Stat3, Tgfb1, Wnt3a,5a
Mm_Focal_Adhesion WP85_41365	2.83E-05	77	191	Bcl2, Capn1, Ccnd1,2,3, Egfr, Fgr, Gsk3b, Igf1, Map2k1,3,4,6, Mapk1,9,12, Myl6, Pten, Rapgef1, Rhob, Rock1,2, Vegfa, c
Mm_Wnt_Signaling Pathway and Pluripotency WP723_41353	3.32E-05	47	97	Ccnd1,2,3, Crebbp, Ctbp1, Ctnnb1, Ep300, Fzd6,8, Gsk3b, Lrp6, Mapk9,10, Sox2, Tcf1,3,4, Wnt2b, 3,3a,5a,5b,7b,9b,10b,16