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14. ABSTRACT

It is unclear why some individuals develop PTSD while others do not. Pre-existing biological differences among individuals may predispose some and not others to develop PTSD. We are seeking to identify such biological differences using an existing rat model for PTSD where exposure to a predator stress scent (PSS) results in extreme behavioral responses for 25% of animals (EBR) thought to be analogous to "PTSD," while 25% of the animals show minimal behavioral responses (MBR). Our objectives for Experiment 1 were to identify genes showing altered mRNA expression in blood and brain areas implicated in PTSD that relate to individual differences in fear reactivity. Our goal in Experiment 2 was to examine the prophylactic effects of exogenous glucocorticoids on the behavioral and gene expression effects of stress exposure. We have completed the behavioral work for Exp.1 and Exp.2 and successfully produced rats with the behavioral phenotypes that were required for our gene expression aims. Gene expression profiling of Exp.1 has been completed on all brain samples and on a portion of the blood. Among others, differential expression of genes (e.g. FKBP5) regulated by the glucocorticoid-receptor (GR) underlies the behavioral differences. The small overlap in gene expression between different tissue and gender demonstrated the existence of distinct tissue specific susceptibility pathways in males and females. Gene expression profiling of the samples of Exp.2 is ongoing.

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Posttraumatic stress disorder, animal models, gene expression

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Final Report for Army Award W81XWH-08-2-0021, entitled "Molecular Mechanisms Underlying Individual Differences in Response to Stress in a Previously Validated Animal Model of PTSD"

INTRODUCTION:

Molecular mechanisms associated with susceptibility to trauma are essential in explaining observations in the neurobiology of PTSD that have been difficult to explain using traditional stress concepts. Although there have been some advances in the identification of genes differentially regulated in PTSD, methodological constraints have limited interpretation of these findings, e.g., variation in the type and magnitude of trauma exposure, human genetic variation, tissue specificity issues (e.g., blood vs. brain). Animal models of PTSD are useful in delineating some of these issues; in this study we used a unique model of PTSD with ecological validity (Cohen and Zohar 2004; Cohen, Zohar et al. 2004). In Experiment 1, adult rats were exposed to a cat urine predator scent stressor (PSS) for 10 min, mimicking a brief, intense threatening experience. Interestingly, rats respond heterogeneously to this type of traumatic stress behaviorally and physiologically, similar to human stress response variability. The two extremes were studied, that is, those clearly vulnerable (or PTSD-like) and those clearly resistant by behavioral criteria. In a subsequent experiment (Experiment 2) we also examined the prophylactic effects of exogenous glucocorticoids (de Quervain 2006; Schelling, Roozendaal et al. 2006) on the behavioral and gene expression effects of stress exposure.

BODY:

As outlined in the statement of work (SOW), the following activities were performed. A summary of pertinent results is also presented.

1. **Compliance and training.**
2. **Fear paradigm was established.**
3. **Collection and processing biological samples in preparation for analyses.**

The current project was funded in 2008 and as stated in the original plan, the first year of the grant was dedicated to obtaining local regulatory approval, establishing the fear paradigm, behavioral phenotyping, and collecting and processing the biological samples. The plan was to conduct these processes in the animal facility at the JJP Bronx VAMC. However, during this process, we learned that Dr. Hagit Cohen, who developed the PSS protocol, offered to conduct the behavioral phenotyping in her laboratory in Israel and send the biological samples to New York for processing. The budget was revised to reflect this change. Shipping costs were paid, but no DOD funds were used for animal experiments.

Behavioral data were presented previously (Annual report 2009) for Experiment 1. Briefly, in Experiment 1, Sprague-Dawley rats were exposed to PSS. The outcome measures included behavior in an elevated plus-maze and acoustic startle response 7 days after the exposure. Cut-off behavioral criteria classified exposed animals according to behavioral responses in both paradigms as those with 'extreme behavioral response' (EBR) and 'minimal behavioral response' (MBR), with unexposed rats as controls. The assessment of behavioral phenotypes was performed in two steps. We first performed a preliminary data assessment to verify that PSS had a significant behavioral effect on the exposed rats as a group, and elicited a range of individual responses. Cutoff behavioral criteria (CBC) incorporating fearful behavior on the elevated plus maze and non-habituated/exaggerated acoustic startle responses were then used to divide the PSS-exposed rats into three behavioral phenotypes: extreme behavioral responders (EBR), partial behavioral responders (PBR) and minimal behavioral responders (MBR). To maximize resolution and minimize false positives extreme responses on both the EPM and ASR were required for inclusion in the EBR group (anxious, fearful and hyper-vigilant, i.e. PTSD-like symptoms), whereas a negligible degree of response to both was required for inclusion in the MBR group. The validity of the criteria have been previously verified by ascertaining that the vast majority (>90%) of unexposed control animals conform to the criteria for MBR (unaffected by test procedures) and less than 3%, meet the criteria for EBR (Cohen et al., 2006). In Experiment 2, a second cohort of rats, the exposed and non-exposed rats with were treated with high-dose corticosterone (CORT) one hour following the PSS exposure. Behavioral results obtained from the both experiments confirmed that the stress paradigm reliably produced three distinct behavioral phenotypes in both male and female rats.

4. Brain microdissection is performed for brain areas: LA, CE, vmPFC, dmPFC.
5. RNA is extracted from rat brain (LA, CE, dmPFC, and vmPFC).
6. RNA amplification and gene expression profiling.

Following completion of the behavioral work described above, animals were sacrificed and brain tissues were collected from 3 different brain areas: amygdala, anterior cortex and hippocampus. Brain tissues from the three different regions were collected, immediately kept in RNAlater solution (Ambion) following the manufacturer's manual to stabilize RNA, and then stored at − 80 °C. RNeasy Mini Kits (Qiagen) was used to isolate total RNA. On-column DNase digestion was used to remove DNA contamination. The purity and quality of the extracted total RNA were evaluated using the RNA 6000 LabChip kit and Agilent 2100 Bioanalyzer (Agilent Technologies). RNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies). High quality RNA with RNA integrity numbers (RINs) greater than 8.0 was used for the microarray experiments.

At the time of the sacrifice, 2.5 ml whole peripheral blood from each subject was also collected in PAXgene tubes. Blood samples were incubated at room temperature for 4 hours for RNA stabilization and then stored at − 80 °C. RNA was extracted from whole blood using the PAXgene Blood RNA System Kit following the manufacturer's guidelines. RNA purification procedures followed the manufacturer's manual with the addition of on-column DNase digestion. Since there is a predominant presence of globin transcript in whole blood samples that constitute approximately 70% of mRNA, the globin depletion method was added to remove globin interference in the blood total RNA: the GLOBINclear™ Mouse/Rat kit (Ambion) was used to deplete globin mRNA from the blood total RNA samples

In brief, 2 μg of total RNA from whole blood was mixed with a biotinylated Capture Oligo Mix in hybridization buffer. The mixture was incubated for 15 minutes to allow the biotinylated oligonucleotides to hybridize with the globin mRNA species. Streptavidin magnetic Beads were then added to capture the globin mRNA. The magnetic beads were then pulled to the side of the tube with a magnet and the RNA, depleted of the globin mRNA, was transferred to a fresh tube. The RNA was further purified using a rapid magnetic bead-based purification method as suggested by the manufacturer. The RNA concentration and purity were determined. Only high quality RNA with RNA integrity numbers (RINs) greater than 8.0 was used for the microarray experiments.

The above procedures were completed for samples from Experiment 1 and 2.

7. Microarray data is analyzed.

Gene expression profiling for samples from Experiment 1 was performed at the Mount Sinai Core facility using the Illumina Rat Ref-12 Expression BeadChip Kit (Illumina) for genome-wide expression analysis. The BeadChip contains 21,910 probes selected primarily from the NCBI RefSeq database (Release 16). Illumina TotalPrep RNA Amplification Kit (Ambion) was used to do reverse transcription and in vitro transcription to synthesize cRNA from 200 ng RNA per sample. Briefly, double-stranded cDNA was synthesized using T7-oligo (dT) primers, followed by an in vitro transcription reaction to amplify cRNA while biotin was incorporated into the synthesized cRNA. After purification, the biotinylated cRNA was quantified using a NanoDrop spectrophotometer and the size distributions of cRNA assessed using Bioanalyzer. Then, 1.5μg biotinylated cRNA was hybridized to Rat Ref-12 Arrays (Illumina). The hybridization, washing, and scanning were performed according to the manufacturer's instructions. Microarray images were extracted automatically during the scanning. BeadStudio (Illumina) was used to normalize raw microarray intensity data.

Gene expression profiling for samples collected from animals in Experiment 2 is ongoing and we expect to complete the data analysis by the end of 2012. Gene expression profiling for samples collected from animals in Experiment 2 is ongoing and will be completed shortly. One reason for the delay is that the Illumina chip was no longer available for the rats, and we had to switch to an alternative platform, which required some realignment

on our part because the new method was more expensive. We have located other internal funds to support this effort.

8. Pathway and Functional enrichment analysis.

Data quality control and analysis of Experiment 1 was completed using R and LIMMA (Linear Models for Microarray Data; as included in MeV), respectively. These methods are being used for clustering analysis and for detection of differentially expressed genes across the different animal groups. Pathway analysis was performed with Ingenuity. Microarray analysis of Experiment 1 revealed significant gene expression differences related to post-trauma phenotype. There was a small overlap in gene expression between tissue and gender. Within the differential expressed genes, genes regulated by the GR (e.g. FKBP5, Per-1, NPY) was particularly over-represented indicating that glucocorticoid regulation is involved in vulnerability and resilience to trauma (Figure 1).

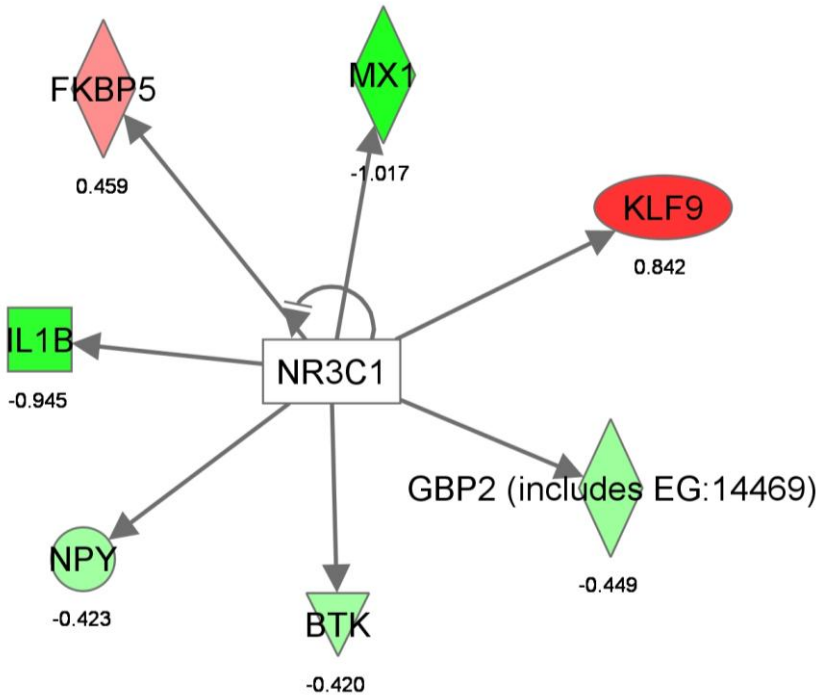

Figure 1. Blood top GR (NR3C1)-regulated genes in EBR vs. MBR blood differential gene expression. Color indicates induction (red) or repression (green). Numbers represent the log ratio between the two groups. Square shape indicates cytokines.

The observed gene expression profiles might be also associated with the overrepresentation of discrete functional biological clusters and pathways (e.g., MAPK signaling, circadian rhythm). The findings from Experiment 1 provider further justification for conducting Experiment 2, in which the influence of post-trauma CORT administration on gene expression will be further clarified.

9. Confirmation of changes in gene expression using real time qPCR.

qPCR validation will be performed after all bioinformatics analyses of both experiments are finished.

KEY RESEARCH ACCOMPLISHMENTS:

- Animal subcommittee approval for the study was sought and obtained.
- Use of the fear paradigm was established and the usefulness of this paradigm for our studies was demonstrated.
- The behavioral work for Experiment 1 and Experiment 2 was completed.
- Biological samples from Experiment 1 and Experiment 2 were obtained and brain dissection was performed.

- RNA extraction and gene expression analysis were performed on the brain and blood samples from Experiment 1 and is ongoing for Experiment 2. We have not applied for a no-cost extension because funds have been spent; the analysis from Experiment 2 is still ongoing and is supported by internal funds.

- Rat genome-wide expression was observed in both genders in brain and blood samples divided into three groups: EBR (PTSD-like), MBR (well-adapted) and CON (control).

- Bioinformatics analysis has shown a list of differentially expressed genes between peripheral tissues and genders. The presence of GR-regulated genes was pronounced. Ingenuity Pathway Analysis is ongoing.

- We will repeat the same measurements and analyses in tissue extracted from rats with post stress CORT-treatment.

- We will have the opportunity to compare the results obtained here in the animal model with our results in PTSD patients in our ongoing DOD-funded studies.

REPORTABLE OUTCOMES:

1. EBR male and EBR female rats differ with respect to gene activity of glucocorticoid related genes (see diagram), which parallels findings from genome wide analyses in men and women with PTSD
2. Glucocorticoid related gene expression underlies susceptibility to behavioral phenotypes linked with PTSD in an animal model
3. Cortisol treatment alters the behavioral phenotype and corresponding gene expression.

CONCLUSION:

Genome-wide gene expression analysis from a unique animal model of PTSD with ecological validity is complete and will help understanding of molecular mechanisms of trauma transduction and susceptibility to trauma effects. Bioinformatics analysis has shown that increased GR-regulated gene expression in blood and brain in the aftermath of traumatic stress contributes to the vulnerable phenotype in this PSS rat behavioral model. Distinct tissue-specific pathways for PTSD-like behaviors exist for males and females.

REFERENCES:

Cohen, H. and J. Zohar (2004). "An animal model of posttraumatic stress disorder: the use of cut-off behavioral criteria." Ann N Y Acad Sci **1032**: 167-178.

Cohen, H., J. Zohar, et al. (2004). "Setting apart the affected: the use of behavioral criteria in animal models of post traumatic stress disorder." Neuropsychopharmacology **29**(11): 1962-1970.

de Quervain, D. J. (2006). "Glucocorticoid-induced inhibition of memory retrieval: implications for posttraumatic stress disorder." Ann N Y Acad Sci **1071**: 216-220.

Schelling, G., B. Roozendaal, et al. (2006). "Efficacy of hydrocortisone in preventing posttraumatic stress disorder following critical illness and major surgery." Ann N Y Acad Sci **1071**: 46-53.