Genetic Profiling of the Hippocampus during Peripheral Chronic Inflammatory Pain

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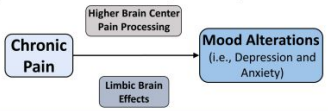
Introduction

Clinical studies have shown a high co-morbidity between different chronic pain conditions and major depressive disorder. The exact brain mechanisms that connect these two neurological illnesses are still largely unknown; however, it is thought that chronic pain may produce negative effects on different limbic brain regions similar to chronic stress.

Here, we used a genome-wide microarray analysis to examine the genetic profile of the hippocampus, a limbic region that regulates mood and stress responses, from male rats exposed to 21 days of inflammatory pain. Bioinformatic gene network/canonical pathways analyses have identified significantly dysregulated genes with known roles in either neuroinflammation or neurodegenerative processes. Lipocalin-2 (Lcn2) or NGAL was identified as one of the highest upregulated genes (~ 2-fold) within the hippocampus during chronic pain state. Lcn2/NGAL is an iron-related protein with roles in innate immune response and cell differentiation/maturation that was recently implicated in regulation of emotional behaviors and cognitive function through regulation of neuronal excitability and dendritic spine formation/maturation. Besides the hippocampus, robust increases in Lcn2/NGAL mRNA were also observed within the prefrontal cortex (PFC) and anterior cingulate cortex (ACC), as well as in the brains of female rats exposed to the same pain paradigm.

Overall, the results of this study continue to strengthen the idea that dysregulation of genes involved in neuroinflammatory and neurodegenerative processes in the hippocampus and other limbic brain areas may be involved in the development of mood disorders during the chronic pain state.



Methods

Inflammatory Pain Model

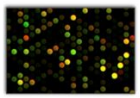
Male and female (6-8 weeks old; 150-350g) Sprague-Dawley rats (Charles River, Wilmington, MA) were age and weight matched and pair-housed with *ad libitum* access to food and water. Rats in the pain group were administered a 50µL subcutaneous injection of Complete Freund's Adjuvant (Sigma Chemical Co., St. Louis, MO) into the plantar surface of the left hind paw, while animals in the control group received a sham needle injection. To model chronic inflammatory pain state, both male (n=8; **Figure 1A**) and female (n=10; **Figure 3A**) were exposed to 21 days of CFA injections administered on days 0, 7, and 14 of the experimental paradigm. For the acute inflammatory pain model, male rats were exposed to a single CFA injection for 24 hours (**Figure 2A**).

Chronic Oral CORT Administration

The rats were provided with water containing 50ug/ml of CORT for 2 weeks. A freshly prepared solution of CORT was provided every 3 days. Daily dosages of CORT were calculated using overnight water consumption values and body weight. 5-7mg/kg was considered the minimum effective dosage. After the initial 2 weeks, the CORT was tapered off with 3 days of 25ug/ml CORT followed by 3 more days at 12.5 ug/ml. The rats were returned to regular drinking water for 3 days to clear the system of exogenous CORT before injection and sacrifice.

Hippocampal Microarray Analysis

Whole genome expression GE two-color microarrays (Agilent Technologies Inc., Santa Clara, CA) were used to analyze transcriptional changes in the hippocampus of rats exposed to 21 days of chronic inflammatory pain (CFA). Raw microarray data was further analyzed utilizing GeneSpring 13.1.1. software (Agilent Technologies Inc.) for identification of significantly dysregulated genes. Statistical significance (p-value < 0.05) was determined using an adjustment for false discovery rate (FDR).



- **Hippocampus from male 21-day CFA cohort (n=8)**
- **Genome-wide hippocampal microarray of the chronic pain state**
  - ***45,598 gene probes in total***
- **Two color competition array**
  - ***Cy3 (green)= Control***
  - ***Cy5 (red)= Pain***

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Expression of target genes was analyzed using a hot-start SYBR Green qPCR method. Fold changes in gene expression were quantified and analyzed using the ΔΔCt method, normalizing to the expression of a house-keeping gene (i.e., HMBS or GAPDH).

Results

Chronic Pain Induces Alterations in Expression of Hippocampal Genes

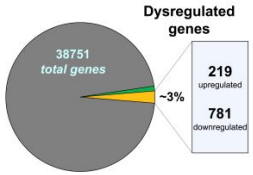


Figure 1. Genome-wide Dysregulation within the hippocampus in the chronic pain state. A total of 38,751 genes (including splice variants) were probed for in the genome-wide microarray analysis. Based on FDR-adjusted p-values (p<0.05) and fold change (>1.3 and <-1.5), 1029 genes (~3%) are significantly dysregulated – a total of 219 genes (green) are upregulated and 781 genes (yellow) are downregulated. Additional gene expression summary of the hippocampus and prefrontal cortex.

Gene Name	Symbol	Fold Change	p-value
Neurodegeneration			
S100 Calcium Binding Protein A9	S100a9	2.31	0.028
Chemokine (C-C motif) ligand 5	CCL5	2.28	0.016
Bcl2-related protein A1	Bcl2a1	2.08	0.016
Fibroblast growth factor 23	FGF23	1.88	0.016
Rho guanine nucleotide exchange factor 15	Arhgef15	1.94	0.016
Intraflagellar transport 57	Ift57	1.71	0.016
Lysyl oxidase	Lox	1.69	0.025
Neurogenesis, Cell Cycle			
Mis18 kinetochore protein A	Mis18a	0.25	0.026
Sodium/Choline cotransporter 7	Slc5a7	0.48	0.038
NOC80 Kinetochore complex component	Ndc80	0.48	0.016
ATPase, type 8A, member 2	Atp8a2	0.53	0.037
Desert Hedgehog	Dhh	0.55	0.016
Calmodulin binding transcription activator 1	Camta1	0.56	0.016
Aurora Kinase C	AurkC	0.56	0.040
Establishment of sister chromatid cohesion 2	Esco2	0.57	0.016
Isoprenoid synthase domain containing	Ispd	0.57	0.213
Fanconi anemia, complementation group A	Fanca	0.57	0.016
Gene Name	**Symbol**	**Fold Change**	**p-value**
Pro-Inflammatory			
Granzyme A	Gzma	2.76	0.001
Granzyme K	Gzmk	2.49	0.016
Lipocalin-2	Lcn2	2.26	0.009
Immunoglobulin Superfamily 6	Igsf6	2.20	0.016
Solute Carrier Organic Anion 2a1	Slco2a1	2.34	0.016
Anti-Inflammatory			
Myocardin	Myocd	0.41	0.016
Integrin alpha L	Itgal	0.49	0.019
Purinergic receptor 5	P2RX5	0.57	0.016
NADPH oxidase 4	Nox4	0.51	0.018
Forkhead box A1	Foxa1	0.72	0.015

Figure 2. Chronic pain evokes hippocampal dysregulation of inflammatory- and neuronal morphology function related genes. Genome-wide microarray analysis from the contralateral hippocampus of rats exposed to chronic inflammatory pain show significant dysregulation in genes that are known to have a function in inflammatory processes and neuronal function (n=8). There is an upregulation of pro-inflammatory and neurodegeneration genes and a downregulation of anti-inflammatory and neurogenesis genes.

Dysregulated Genes within the Hippocampus and Prefrontal Cortex

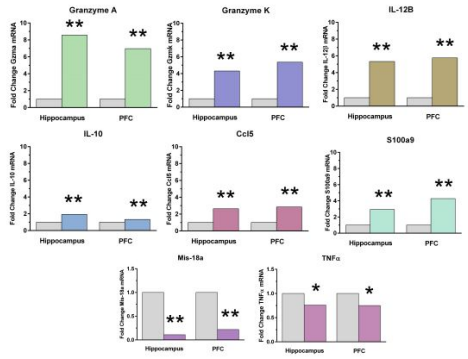


Figure 3. Gene expression summary of the hippocampus and prefrontal cortex of male and female rats exposed to 21 days of chronic inflammatory pain. Gene expression of Gzma, Gzmk, IL-12B, ITGAL, IL-10, Ccl5, S100a9, Mis18a, and TNFa in male and female rats exposed to 21 days of chronic inflammatory pain (CFA) p<0.05*, p<0.01**.

Effects of Chronic Pain on Limbic Lcn2 Activity in Male and Female Rats

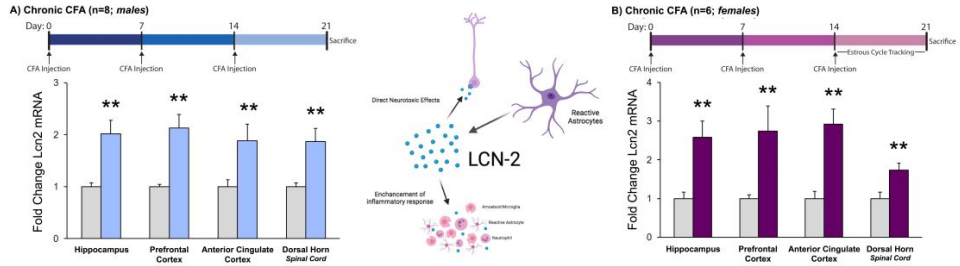


Figure 4. Expression of Lcn2 gene in different limbic brain areas and spinal cord of male (A) and female (B) rats exposed to 21 days of peripheral inflammatory pain. Levels of Lcn2 mRNA were analyzed in the contralateral hippocampus, prefrontal cortex (PFC), anterior cingulate cortex (ACC) and dorsal horn of the spinal cord. Fold changes are expressed as mean ± s.e.m. (n=6-8) after normalization to housekeeping genes, p<0.01**.

Effects of Chronic CORT on Hippocampal Lcn2 Activity in Male and Female Rats

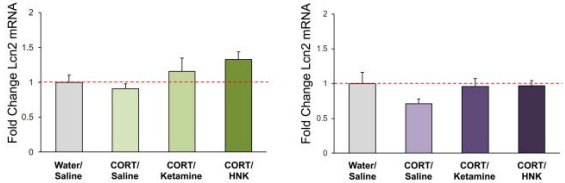


Figure 5. Expression of Lcn2 in the hippocampus of male and female rats exposed to 21 days of corticosterone (CORT) in drinking water. Levels of Lcn2 mRNA in male (**A**) and female (**B**) rats were analyzed in the contralateral hippocampus using qPCR. Fold changes are expressed as mean ± s.e.m. (n=6) after normalization to housekeeping genes (i.e., β-Actin). Analysis of variance (ANOVA) indicated no significance.

Effects of Acute Pain on Limbic Lcn2 Activity in Male Rats

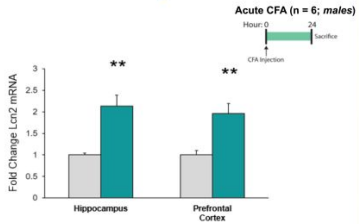


Figure 6. Expression of Lcn2 gene in the contralateral hippocampus and prefrontal cortex of male rats exposed to 24 hr of peripheral inflammatory pain. Fold changes are expressed as mean ± s.e.m. (n=5-6) after normalization to housekeeping genes (i.e., GAPDH, β-Actin). ** p<0.01 compared to naïve controls (*Student's t-test*).

Summary

- Chronic pain altered expression of genes involved in ***immuno-inflammatory*** and ***neurodegenerative*** processes within the rat contralateral hippocampus and prefrontal cortex (PFC)
  - *Genome-wide microarray analysis of the hippocampus identified a total of **219 up-regulated** and **781 down-regulated** genes*
  - *Dysregulation of several genes of interest (e.g., **Gzma**, **Gzmk**, **IL-12β**, **ITGAL**, **Ccl5**, **TNFa**, **IL-10**, **Mis18a**, and **S100a9**) was confirmed using qPCR in both male and female rats*
- Significant increases in Lcn2 activity within the brain areas involved in affective/emotional component of pain in both male and female rats
  - ***Contralateral hippocampus***, ***prefrontal cortex*** (PFC) and ***anterior cingulate cortex*** (ACC)
  - Significant upregulation of Lcn2 within the ***dorsal horn of the spinal cord***, a major neurophysiological component of the sensory component of pain
- **Robust transcriptional alterations in the limbic system during the chronic pain state suggest that pro-inflammatory and neurodegenerative processes in these brain areas may be involved in the development of depression and other mood disorders in chronic pain patients**

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