ARTICLES

Microarray Analysis of High-Dose Recombinant Erythropoietin Treatment of Unilateral Brain Injury in Neonatal Mouse Hippocampus

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ABSTRACT: Recombinant human erythropoietin (rEpo) is neuroprotective in neonatal models of brain injury. Proposed mechanisms of neuroprotection include activation of gene pathways that decrease oxidative injury, inflammation, and apoptosis, while increasing vasculogenesis and neurogenesis. To determine the effects of rEpo on gene expression in 10-d-old BALB-c mice with unilateral brain injury, we compared microarrays from the hippocampi of brain-injured pups treated with saline or rEpo to similarly treated sham animals. Total RNA was extracted 24 h after brain injury and analyzed using Affymetrix GeneChip Mouse Exon 1.0 ST Arrays. We identified sex-specific differences in hippocampal gene expression after brain injury and after high-dose rEpo treatment using single-gene and gene set analysis. Although high-dose rEpo had minimal effects on hippocampal gene expression in shams, at 24-h post brain injury, high-dose rEpo treatment significantly decreased the proinflammatory and antiapoptotic response noted in saline-treated brain-injured comparison animals. (*Pediatr Res* 65: 485–492, 2009)

Hypoxia-ischemia (HI) is the most common cause of brain injury in the neonate. HI results in a complex cascade of events that includes energy failure, ionic disruption, inflammation, free radical generation, and subsequent cell death. The response to HI-induced brain injury is region-specific and developmentally determined (1). Treatment options are currently limited. Understanding the specific changes in gene expression associated with HI may allow for the formulation of new treatment strategies.

High-dose recombinant erythropoietin (rEpo) has neuroprotective effects in neonatal models of brain injury (2–4). The mechanism of rEpo neuroprotection is complex and not fully understood. There is evidence that both neuronal (direct) and nonneuronal (indirect) factors are involved. Direct effects include Epo receptor-mediated increases in antiapoptotic gene expression (5) and promotion of neurogenesis (6–8). Indirect effects include an increase in antioxidant activity in astrocytes (9,10) and a decrease in the inflammatory response to injury (11,12). Epo also increases the utilization of iron for erythropoiesis, which may decrease the oxidative consequences of HI injury. The vasculogenic effects of rEpo may also improve long-term healing from such an injury (8,13).

Microarray technology is a powerful tool suitable for the elucidation of mechanisms underlying complex biologic processes such as rEpo neuroprotection. Recognizing that gene expression-based microarray technologies have rapidly advanced and that new bioinformatics software provides improved normalization, transformation, visualization, cluster analysis, and annotation, we now extend our examination of rEpo-induced changes in hippocampal gene expression in the context of brain injury. In our previous study, we examined pooled triplicate samples from three brain regions, at three time points, with male and female samples combined (14). We now focus on hippocampal gene expression 24 h after HI-induced injury using individual RNA samples from six replicates. This allows for assessment of interanimal variability as well as gender differences in response to injury and treatment. The goals for this study were first to define the pattern of hippocampal gene expression evident 24 h after HI, then to determine specific changes in expression associated with rEpo treatment, and finally, to compare the responses between males and females. These experiments were necessary to generate a more precise understanding of the neuroprotective mechanisms underlying neonatal rEpo therapy.

METHODS

Animal methods. All experimental protocols were approved by the Animal Care and Use Committees at the University of Washington in accordance with U.S. National Institutes of Health guidelines. Animals were housed with a 12-h light/dark cycle and fed *ad libitum*. All pups were weighed and distributed to create weight-matched litters ($n = 5–7$/dam).

Abbreviations: EI, brain injured, rEpo-treated; **GSA,** gene set analysis; **HI,** hypoxia-ischemia; **I,** brain injured, vehicle-treated; **rEpo,** recombinant human erythropoietin; **S,** sham, vehicle-treated

Treatment groups. Four groups of BALB-c mice (Harlan, Kent, WA) were studied: sham-vehicle (S) $n = 6$, sham-rEpo (E), $n = 6$, brain-injury-vehicle (I), $n = 28$, and brain-injury-rEpo (EI), $n = 24$. For the microarray portion of the study, six animals were studied per group.

Brain injury. Using 2.5% isoflurane anesthesia, the right carotid artery of 10-d-old mice were cauterized. Animals were recovered for 30 min and then returned to their dam for 1 h. They were next exposed to 8% oxygen $\times$ 15 min alternating with 100% oxygen $\times$ 10 min for a total of 45 min of hypoxia and 20 min of hyperoxia at 35°C. Animals received either vehicle or rEpo 5000 U/kg (Procrit, Amgen, Thousand Oaks, CA). The first injection was given 30 min after the completion of hypoxia and the second was given 18 h later. Pups were euthanized 24 h after injury by an overdose (75 mg/kg) of Euthasol i.p. (Delmarva Labs, Midlothian, VA).

Assessment of brain injury. Gross brain injury in the hemisphere ipsilateral to ligation was assessed on a five point ordinal scale (0–4) as follows: 0 = no injury, 1 = mild injury with < 25% lesion of ipsilateral hemisphere, 2 = 25–50% lesion, 3 = 51–75%, and 4 = ≥75% injury. Groups were compared using a two-tailed t test.

In a subset of animals, 2,3,5-triphenyltetrazolium chloride (TTC) staining was used to document subcortical brain injury on 2-mm coronal brain sections cut using a mouse brain grid. Sections were incubated in 2% TTC in 0.9% PBS for 30 min at 37°C, fixed with 10% formalin, rinsed in PBS, and photographed.

RNA methods. RNA from the right hippocampus was isolated following manufactures' directions (Cartagen Molecular Systems, San Carlos, CA). The quality of RNA was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only RNA samples with appropriate size distribution, quantity, and an A260:A280 ratio of 1.8–2.1 were used for analysis.

Microarray processing. The manufacturer's protocols for the GeneChip platform by Affymetrix (Santa Clara, CA) were used. Methods included synthesis of first- and second-strand cDNAs, the purification of double-stranded cDNA, synthesis of cRNA by *in vitro* transcription, recovery and quantitation of biotin-labeled cRNA, fragmentation of this cRNA and subsequent hybridization to the microarray slide, posthybridization washings, and detection of the hybridized cRNAs using a streptavidin-coupled fluorescent dye. Hybridized Affymetrix arrays were scanned with an Affymetrix Gene-Chip 3000 scanner. Image generation and feature extraction were performed using Affymetrix GCOS Software.

Statistical analysis and data normalization for Affymetrix Mouse Exon 1.0 ST Arrays. Raw microarray data were processed with Bioconductor (15), and exonic probe sets (16) were analyzed. Probes were normalized with Robust Multi-Array (RMA) (17,18). From the normalized data, genes with significant evidence for differential expression were identified using the Bioconductor limma package (19). The p values were calculated with a modified t test in conjunction with an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. The p values were adjusted for multiplicity with the Bioconductor package q-value (20), which allows for selecting statistically significant genes while controlling the estimated false discovery rate.

Single gene analysis. We defined groups of genes which were most protected by rEpo treatment and those which were least protected. Conceptually, the most protected genes are genes whose expression is highly changed by brain injury, but whose expression in the presence of rEpo plus conditions, which stimulate brain injury, revert to baseline (sham) expression levels. Least protected genes are genes whose expression changes with brain injury and for which rEpo has little or no effect. These concepts are defined mathematically by comparing our four treatment groups as shown in Table 1. Baseline gene expression in the sham vehicle-treated group (S) is denoted as μ. The addition of rEpo to sham is denoted as $\mu + \alpha$, where α is the additional effect of rEpo. The addition of injury (I) to baseline gene expression is denoted as $\mu + \beta$, where β is the additional effect of injury. Finally, in the rEpo-treated brain-injured group (EI), which has effects of both rEpo and injury, we denote the expression level as $\mu + \alpha + \beta + \gamma$, where γ is the interaction effect of rEpo and injury, which is the additional effect of rEpo and injury beyond what we would expect from rEpo or injury alone.
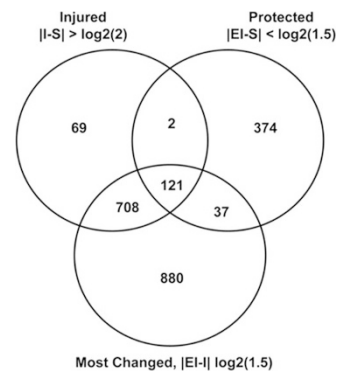

Figure 1. Venn Diagram. Venn diagram showing most protected genes by comparing the three contrasts and genes they have in common (p values <0.05).

1. To calculate the effect of injury, β, we look at the difference between the two groups sham and injury, S and I. The differential gene expression given by the contrast I vs. S (|I–S| > log2(fold) and $p < 0.05$, or $\beta > log2(fold)$) tells us which genes are most changed by injury alone (Fig. 1, top left circle).
2. For rEpo-treated injury, there are genes that are unchanged compared with the sham-vehicle-treated group. For this group of genes the sum $\alpha + \beta + \gamma$ is nearly zero. The differential gene expression for this group is given by the contrast EI vs. S (|EI–S| < log2(fold) and $p < 0.05$) and tells us which genes did not change much between the sham group and the rEpo-treated injury group (Fig. 1, top right circle).
3. For rEpo-treated injury, there are the injured genes that showed large differential gene expression changes. These genes may have been protected by the rEpo treatment to a large degree, but not enough so that the rEpo treatment made the genes look as if they were not injured. The differential gene expression given by the contrast EI vs. I (|EI–I| > log2(fold) and $p < 0.05$) tells us which genes changed the most between the injury group and rEpo-treated injury group (Fig. 1, bottom circle).

Gene set analysis. To investigate categories of genes where the constituent genes show coordinated changes in expression over the experimental conditions, we used gene set analysis (GSA), a type of biologic category analysis (21,22). GSA software is available as R code (21,23) (http://www-stat.stanford.edu/~tibs/GSA/) to calculate separate gene set analyses. GSA considers all the genes in the experiment and allows for the identification of gene sets with strong cross-correlation by boosting the signal-to-noise ratio, making it possible to detect modest changes in gene expression. We used four gene sets for the GSA: Biologic process, molecular function, and cellular component from gene ontology (24), and the C2 gene set from the Molecular Signature Database (23) (http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html).

RESULTS

rEpo treatment with 5000 U/kg i.p. improved the gross brain injury scores 24 h after brain injury. The mean $\pm$ SD injury scores for rEpo-treated brain-injured animals ($n = 24$) were 1.7 $\pm$ 1.8 with a median of 1, compared with vehicle-treated brain-injured ($n = 28$), 2.7 $\pm$ 1.9, median of 3.8 ($p < 0.01$). There were no differences between males and females. In the vehicle-treated animals, injury scores for males and females were 2.6 $\pm$ 2.1 and 2.8 $\pm$ 1.7, respectively. The rEpo-treated males and females had scores of 1.7 $\pm$ 1.9 and 1.6 $\pm$ 1.8, respectively. Gross brain injury scores tended to underestimate brain injury, because some animals demon-

Table 1. *The four treatment groups for the Epo-treated injury experiment*

	Injury	
	No	Yes
rEpo treatment		
No	Sham (S) μ	Injury (I) $\mu + \beta$
Yes	Sham + rEpo (E) $\mu + \alpha$	rEpo + Injury + rEpo:Injury (EI) $\mu + \alpha + \beta + \gamma$

strated subcortical brain injury visualized with TTC that was not apparent grossly. In brains that were sequentially sliced and stained from anterior to posterior, it was noted that brain injury occurred most commonly in the posterior cortex of the ipsilateral hemisphere. Weight loss correlated with brain injury score, $p < 0.001$.

Single gene analysis. Neonatal HI exposure altered hippocampal gene expression. Mean gross brain injury scores of animals used for microarray analysis were 3.9 for vehicle-treated brain injury, 0.4 for rEpo-treated brain injury, and 0 for all sham groups. Nine hundred genes in the hippocampus changed expression after brain injury by at least 2-fold, with $p < 0.05$ (I vs. S). Functional annotation is available for 800: 159 were down-regulated and 641 were up-regulated relative to shams. Examples of genes down-regulated by HI included serotonin receptors, neurogenic differentiation 6, G-coupled receptors, GABA receptors, gastrin, glutamate receptors AMPA1, kainite 4, NMDA2A, and solute carrier family genes such as solute carrier family 6 (neurotransmitter transporter). Genes up-regulated by HI included hypoxia-inducible factor 2, annexins 2, 4, and 5, angiopoietin related protein 4, caspases 4, 7, 8, 12, cathepsins B, C, D, L, S, and Z, multiple chemokines, death-associated protein, bag 3, BAX, colony stimulating factors, heat shock proteins 8 and B6, interferons, interleukins, toll-like receptors 1, 2, 4, 7, 13, tumor necrosis factor receptors, heme oxygenase, *c-fos*, and prostaglandin D2 synthase. The magnitude of change in the HI-exposed subset ranged from 5.6-fold for down-regulated genes to 276-fold for up-regulated genes.

We next identified genes which, in the context of brain injury, are most influenced (most protected) by rEpo. Figure 1 is a Venn diagram that shows the number of differentially expressed genes in three comparison groups, and how these groups overlap. The upper left circle (I vs. S) contains the 900 genes that responded to injury alone by changing their transcript levels >2-fold, $p < 0.05$ when compared with shams. The upper right circle (EI vs. S) illustrates the 534 genes whose expression was not different when rEpo-treated brain-injured animals were compared with sham animals (criterion <1.5-fold, $p < 0.05$). The bottom circle (EI vs. I) represents the 1746 genes whose expression changed significantly when rEpo-treated and vehicle-treated brain-injured animals were compared (criterion >1.5-fold, $p < 0.05$). The overlapping regions show the 92%, or 831 genes, which can be considered most protected by rEpo treatment in the context of brain injury. A subset of these genes are shown in Table 2, and the entire set is available in supplementary material, Table S1.

The magnitude of changes in gene expression in each of our conditions is visually represented in the composite ratio intensity plot, Fig. 2. Panel A shows the difference in gene expression when brain-injured vehicle-treated animals are compared with sham-operated vehicle-treated animals. The red dots represent the genes that are changed by more than 2-fold ($p < 0.05$). Panel B shows the treatment effects of rEpo in the brain-injured animals. In this comparison, rEpo-treated brain-injured animals are compared with brain-injured vehicle-treated animals. The genes that were >2-fold changed by injury (Fig. 2, Panel A) are still shown in red; however, most

of them now fall within the expression range of the uninjured animals. Thus, treatment of brain injury with rEpo caused the gene expression to look more like the shams. Panel C shows the effects of rEpo on hippocampal gene expression in the absence of brain injury. As in Panels A and B, the red colored data points track the genes that were changed >2-fold by injury ($p < 0.05$). There are relatively few changes in gene expression caused by rEpo alone; 62 genes were changed >1.5-fold ($p < 0.05$) and functional annotations are available for 39 of these. These rEpo-responsive genes include the following: Annexin A1 (Anxa1), Bone morphogenetic protein 4 precursor (Bmp4), C3a anaphylatoxin chemotactic receptor (C3AR), NFKB activating protein-like (Nkapl), Protein kinase C delta type (Prkcd), and Glycophorin (Gypa).

GSA analysis. Using GSA, we next identified gene sets/pathways with concordant changes in expression in a given experimental condition. We defined most protected gene sets as those whose expression was altered dramatically by brain injury and rEpo prevented or attenuated this change to a large degree making it look like sham.

Table 3 shows selected gene sets enriched in HI hippocampus when compared with shams, whereas Table 4 shows gene sets enriched in sham hippocampus relative to brain-injured animals. Gene sets are ranked based on differences identified in the brain injury to sham comparison (I vs. S); all are $p < 0.05$. The rank scores for the I vs. S comparison are shown in the first column, followed by the p value. For comparison, the ranking of these gene sets in the EI vs. S analyses are also shown, as are their p values. Note that the p values were not significant in the EI vs. S comparison, demonstrating that expression of these gene sets was modified by rEpo treatment. A full listing of gene sets that met our filtering criteria is provided in supplementary material Table S2.

Many of the gene sets enriched after brain injury were related to inflammatory response, and they included neutrophil, monocyte, lymphocyte and T cell lines, cytokine, and chemokine-mediated signaling pathways, and genes important for phagocytosis. Gene sets having to do with growth factor binding; proteoglycan binding, cell-matrix interactions, and cell movement were also over-represented. As expected, gene sets involved with response to hypoxia were also enriched in brain-injured hippocampus. For example, in Figure 3, a heat map of the C2 gene set *Hypoxia_Review* is shown for our four treatment groups. Each column represents the gene expression for an individual animal, with animals grouped by treatment. Each row represents one gene within the gene set. Relative gene expression is denoted by color, with red illustrating gene expression that is higher than the mean in the control reference group, blue indicating the opposite and white indicating no change in expression. Expression levels for each gene are shown relative to the average expression of that gene in sham-vehicle animals. Sham-vehicle-treated animals are shown in the left grouping, followed sequentially by brain-injured-vehicle-treated animals, brain-injured-rEpo-treated animals, and finally, sham-rEpo-treated animals. This heat map shows the variability in gene expression between animals within each treatment group and the differences in gene expression between treatment groups in the context of the gene

Table 2. *Examples of most protected single genes*

| Ensembl ID	I vs. S fold change	Entrez gene	Description	MGI_symbol
ENSMUSG00000015340	8.84	13058	Cytochrome b-245, beta polypeptide	Cybb
ENSMUSG00000032231	8.02	12306	Annexin A2	Anxa2
ENSMUSG00000041548	6.71	80888	Heat shock protein 8	Hspb8
ENSMUSG00000044583	5.53	170743	Toll-like receptor 7	Tlr7
ENSMUSG00000020932	4.66	14580	Glial fibrillary acidic protein	Gfap
ENSMUSG00000004371	4.53	16156	Interleukin 11	Il11
ENSMUSG00000026981	4.24	16181	Interleukin 1 receptor antagonist	Il1rn
ENSMUSG00000029994	4.21	11746	Annexin A4	Anxa4
ENSMUSG00000002289	4.15	57875	Angiopoietin-like 4	Angptl4
ENSMUSG00000014599	4.15	12977	Colony stimulating factor 1 (macrophage)	Csf1
ENSMUSG00000030560	4.00	13032	Cathepsin C	Ctsc
ENSMUSG00000017057	3.93	16164	Interleukin 13 receptor, alpha 1	Il13ra1
ENSMUSG00000006301	3.81	69660	Transmembrane BAX inhibitor motif containing 1	Tmbim1
ENSMUSG00000040663	3.72	56708	Cardiotrophin-like cytokine factor 1	Clcf1
ENSMUSG00000024087	3.68	13078	Cytochrome P450, family 1, subfamily b	Cyp1b1
ENSMUSG00000031304	3.67	16186	Interleukin 2 receptor, gamma chain	Il2rg
ENSMUSG00000026072	3.65	16177	Interleukin 1 receptor, type I	Il1r1
ENSMUSG00000031465	3.31	11601	Angiopoietin 2	Angpt2
ENSMUSG00000006519	3.22	13057	Cytochrome b-245, alpha polypeptide	Cyba
ENSMUSG00000044827	3.10	21897	Toll-like receptor 1	Tlr1
ENSMUSG00000026029	2.95	12370	Caspase 8	Casp8
ENSMUSG00000004891	2.94	18008	Nestin	Nes
ENSMUSG00000030847	2.87	29810	Bcl2-associated athanogene 3	Bag3
ENSMUSG00000039168	2.84	223453	Death-associated protein	Dap
ENSMUSG00000038642	2.78	13040	Cathepsin S	Ctss
ENSMUSG00000021477	2.68	13039	Cathepsin L	Ctsl
ENSMUSG00000024401	2.64	21926	Tumor necrosis factor	Tnf
ENSMUSG00000052837	2.58	16477	Jun-B oncogene	Junb
ENSMUSG00000025076	2.56	12369	caspase 7	Casp7
ENSMUSG00000001020	2.50	20198	S100 calcium binding protein A4	S100a4
ENSMUSG00000040296	2.44	230073	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	Ddx58
ENSMUSG00000024190	2.26	19252	Dual specificity phosphatase 1	Dusp1
ENSMUSG00000020524	−2.08	14799	Glutamate receptor, ionotropic, AMPA1 (α 1)	Gria1
ENSMUSG00000025092	−2.09	73442	Heat shock protein 12A	Hspa12a
ENSMUSG00000025810	−2.17	18186	Neuropilin 1	Nrp1
ENSMUSG00000026158	−2.22	70155	Opioid growth factor receptor-like 1	Ogfrl1
ENSMUSG00000032017	−2.30	110637	Glutamate receptor, ionotropic, kainate 4	Grik4
ENSMUSG00000074939	−2.45	213788	Cholinergic receptor, muscarinic 5	Chrm5
ENSMUSG00000035594	−2.69	110835	cholinergic receptor, nicotinic, α polypeptide 5	Chrna5
ENSMUSG00000059003	−2.73	14811	glutamate receptor, ionotropic, NMDA2A	Grin2a
ENSMUSG00000028004	−3.41	18167	Neuropeptide Y receptor Y2	Npy2r
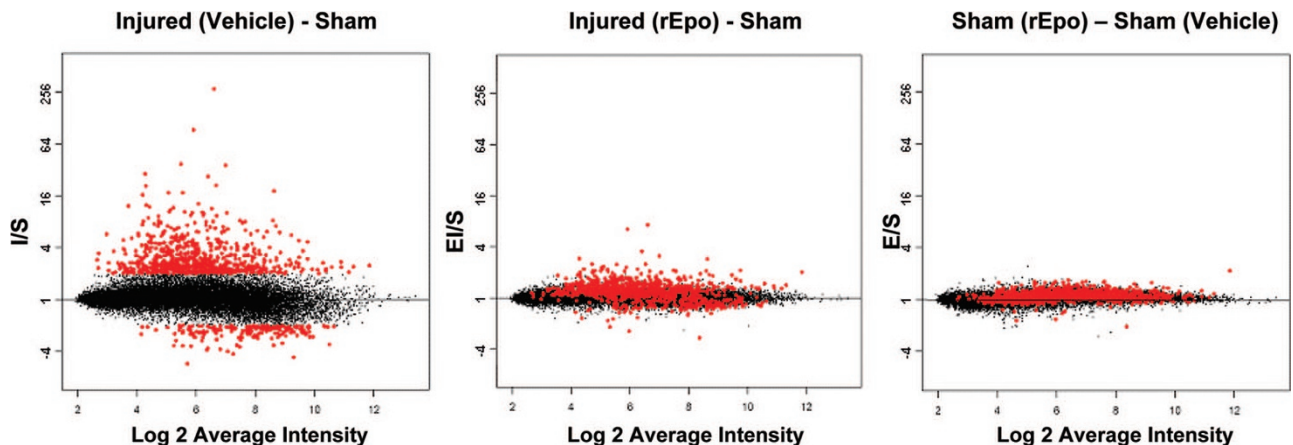
Figure 2. Ratio intensity plots. The horizontal axes show the average log$_2$ signal as a measure of transcript signal intensity, and the vertical axis represents fold changes of transcript signals between the two comparison groups.

set. As expected, vehicle-treated animals exposed to HI show robust changes in gene expression within this gene set. Treatment of brain injury with rEpo restores gene expression to a pattern closer to shams. Sham-rEpo-treated animals are most similar to sham-vehicle-treated animals. In this particular gene set, hypoxic-ischemic brain injury is associated with increased gene

Table 3. *Gene sets enriched in brain injury*

Gene set description	Gene set length	Gene set name	I vs. S score	I vs. S p	EI vs. S score	EI vs. S p
Biological process						
GO:0007044	5	Cell-substrate junction assembly	1.60	0.02	−0.02	0.50
GO:0045637	23	Regulation of myeloid cell differentiation	1.49	0.00	0.35	0.18
GO:0006898	9	Receptor mediated endocytosis	1.39	0.00	0.52	0.10
GO:0001906	8	Cell killing	1.05	0.03	0.64	0.09
GO:0000186	6	Activation of MAPKK activity	0.96	0.00	0.24	0.19
GO:0030851	4	Granulocyte differentiation	0.88	0.00	−0.29	0.31
GO:0019221	25	Cytokine and chemokine signaling pathway	0.79	0.02	0.46	0.07
GO:0030593	11	Neutrophil chemotaxis	0.75	0.03	0.47	0.09
GO:0006968	41	Cellular defense response	0.72	0.00	0.48	0.06
GO:0051252	12	Regulation of RNA metabolism	0.70	0.00	0.09	0.40
GO:0050776	86	Regulation of immune response	0.69	0.01	0.45	0.05
GO:0050671	22	Positive regulation of lymphocyte proliferation	0.63	0.02	0.50	0.12
GO:0031589	44	Cell-substrate adhesion	0.53	0.01	−0.05	0.36
GO:0042060	61	Wound healing	0.44	0.03	0.12	0.36
GO:0016049	69	Cell growth	0.38	0.00	0.17	0.16
Molecular function						
GO:0005035	5	Death receptor activity	1.90	0.01	1.15	0.06
GO:0005178	20	Integrin binding	1.52	0.00	0.15	0.27
GO:0005520	19	Insulin-like growth factor binding	1.35	0.00	0.27	0.25
GO:0008191	6	Metalloendopeptidase inhibitor activity	1.30	0.03	0.45	0.17
GO:0016504	10	Protease activator activity	0.98	0.01	0.24	0.24
GO:0019838	40	Growth factor binding	0.87	0.00	0.28	0.11
GO:0008201	52	Heparin binding	0.69	0.00	0.15	0.28
GO:0005540	16	Hyaluronic acid binding	0.68	0.00	−0.22	0.20
GO:0030247	74	Polysaccharide binding	0.68	0.00	0.14	0.30
GO:0001871	76	Pattern binding	0.66	0.00	0.13	0.32
Cellular component						
GO:0001725	10	Stress fiber	1.22	0.00	−0.57	0.11
GO:0001726	20	Ruffle	0.64	0.00	−0.19	0.27
GO:0005912	49	Adherens junction	0.64	0.00	−0.13	0.27
GO:0005924	22	Cell-substrate adherens junction	0.76	0.02	−0.11	0.34
GO:0005925	21	Focal adhesion	0.78	0.02	−0.12	0.35
GO:0030055	24	Cell-matrix junction	0.66	0.03	−0.17	0.27
GO:0005604	42	Basement membrane	0.60	0.05	0.10	0.33
C2 pathways						
	8	NEUTROPHILPATHWAY	1.45	0.05	0.69	0.12
	14	SA_MMP_CYTOKINE_CONNECTION	1.42	0.00	0.71	0.12
	11	CLASSICPATHWAY	0.98	0.04	0.73	0.16
	22	NFKBPATHWAY	0.93	0.03	0.40	0.15
	9	IL6_SCAR_FIBRO_DN	0.81	0.01	−0.09	0.37
	12	STRESS_P53_SPECIFIC_UP	0.77	0.02	0.58	0.10
	32	IGF1_NIH3T3_UP	0.63	0.00	0.06	0.37
	70	HYPOXIA_REVIEW	0.63	0.00	0.10	0.39
	67	DNMT1_KO_UP	0.54	0.01	0.16	0.21
	33	TOLLPATHWAY	0.54	0.01	0.07	0.36
	24	STRESSPATHWAY	0.43	0.03	−0.18	0.23
	47	TGF_BETA_SIGNALING_PATHWAY	0.42	0.00	0.10	0.29

expression of members including HIF-1α, HMOX1, IGFBP2, IGFBP3, TGFB1, IL-6, Fos, Jun, angiopoietin 2, and NFkB1.

A similar pattern is identified in the gene set named Regulation of Inflammatory Response, with brain-injured animals showing marked up-regulation of transcription for a majority of genes in this family (Fig. 4). Hippocampus from rEpo-treated brain-injured animals shows a pattern of gene expression more similar to the shams, and rEpo treatment of shams does not significantly affect the overall transcription of these particular genes.

Gene sets in which expression was significantly decreased in brain-injured hippocampus relative to uninjured brain had largely to do with cell maintenance and growth. Pathways associated with lipid, phospholipid, and glycoprotein metabolism, amino acid and ion transport, and synaptic transmission were in this category. Expression of these gene sets in rEpo-treated brain-injured animals was more like the shams.

Male/female comparison. To determine whether males respond differently to brain injury or to rEpo treatment than females, we carried out a limited subgroup analysis, comparing three males and three females in each group. In the brain injury *versus* sham (I vs. S) comparison using a 2-fold cutoff with $p < 0.05$, 955 genes were differentially expressed by males, compared with only 820 in females. Males also appeared to respond more to rEpo treatment than did females (EI vs. I contrast) with 2351 genes differentially expressed, compared with 1063 in females (1.5-fold change, $p < 0.05$). Females appeared more resistant to injury, and less affected by rEpo than males (EI vs. S contrast): males 148 genes, females 374.

Table 4. *Gene sets enriched in shams*

Gene set description	Gene set length	Gene set name	I vs. S score	I vs. S *p*	EI vs. S score	EI vs. S *p*
Biological process						
GO:0008306	5	Associative learning	−2.23	0.00	−1.13	0.06
GO:0007214	16	Gamma-aminobutyric acid signaling pathway	−1.34	0.01	−0.43	0.13
GO:0015804	6	Neutral amino acid transport	−1.33	0.00	−0.30	0.25
GO:0016126	21	Sterol biosynthesis	−1.28	0.01	−0.70	0.13
GO:0046488	6	Phosphatidylinositol metabolism	−1.22	0.02	−0.56	0.17
GO:0046928	6	Regulation of neurotransmitter secretion	−1.11	0.02	−0.34	0.27
GO:0006885	13	Regulation of pH	−0.89	0.04	−0.42	0.11
GO:0007270	22	Nerve-nerve synaptic transmission	−0.73	0.00	−0.36	0.15
GO:0050678	10	Regulation of epithelial cell proliferation	−0.56	0.03	−0.11	0.38
GO:0006638	13	Neutral lipid metabolism	−0.54	0.00	−0.08	0.30
GO:0018346	10	Protein amino acid prenylation	−0.47	0.04	−0.43	0.09
GO:0050801	82	Ion homeostasis	−0.43	0.03	−0.21	0.22
GO:0006650	37	Glycerophospholipid metabolism	−0.40	0.04	−0.09	0.36
GO:0000082	20	G1-S transition of mitotic cell cycle	−0.38	0.03	−0.06	0.41
GO:0006644	85	Phospholipid metabolism	−0.35	0.01	−0.06	0.32
GO:0009100	76	Glycoprotein metabolism	−0.32	0.04	−0.14	0.22
GO:0008154	38	Actin polymerization and-or depolymerization	−0.30	0.01	−0.07	0.32
Molecular function						
O:0015491	11	Cation:cation antiporter activity	−1.72	0.01	−1.00	0.06
GO:0004994	5	Somatostatin receptor activity	−1.50	0.05	−0.12	0.43
GO:0005451	6	Monovalent cation:proton antiporter activity	−1.47	0.02	−0.42	0.20
GO:0019209	7	Kinase activator activity	−1.20	0.00	−0.49	0.12
GO:0004993	13	Serotonin receptor activity	−1.10	0.05	−0.41	0.22
GO:0008373	19	Sialyltransferase activity	−0.96	0.00	−0.13	0.30
GO:0030159	5	Receptor signaling complex scaffold activity	−0.89	0.03	−0.57	0.17
GO:0019905	11	Syntaxin binding	−0.86	0.03	−0.18	0.36
GO:0015175	8	Neutral amino acid transporter activity	−0.76	0.04	−0.02	0.47
GO:0005248	11	Voltage-gated sodium channel activity	−0.71	0.03	−0.55	0.07
GO:0005343	17	Organic acid:sodium symporter activity	−0.66	0.04	−0.48	0.12
GO:0016917	31	GABA receptor activity	−0.64	0.05	−0.07	0.38
GO:0051766	7	Inositol trisphosphate kinase activity	−0.55	0.02	−0.10	0.37
Cellular component						
GO:0030173	21	Integral to Golgi membrane	−1.00	0.00	−0.38	0.07
GO:0016529	13	Sarcoplasmic reticulum	−0.87	0.02	−0.38	0.11
GO:0031301	36	Integral to organelle membrane	−0.69	0.00	−0.08	0.32
GO:0030018	21	Z disc	−0.53	0.03	−0.41	0.21
GO:0030176	12	Integral to endoplasmic reticulum membrane	−0.39	0.04	0.37	0.11
C2 pathways						
	11	CHOLESTEROL_BIOSYNTHESIS	−2.21	0.02	−0.99	0.16
	12	GABAPATHWAY	−1.92	0.00	−0.83	0.06
	12	BIOSYNTHESIS_OF_STEROIDS	−1.54	0.02	−0.55	0.18
	26	ST_GRANULE_CELL_SURVIVAL_PATHWAY	−1.00	0.00	−0.28	0.14
	31	ST_ADRENERGIC	−0.73	0.00	−0.48	0.06
	23	CXCR4PATHWAY	−0.68	0.01	−0.49	0.05
	13	HDACI_COLON_CURSUL_DN	−0.68	0.03	−0.33	0.17
	10	EGFR_SMRTEPATHWAY	−0.66	0.02	−0.55	0.09
	18	ST_WNT_CA2_CYCLIC_GMP_PATHWAY	−0.66	0.04	−0.46	0.09

DISCUSSION

The developing brain exhibits unique vulnerabilities that are age-dependent. In neonatal brain, excitatory synapses are denser, the vascular system is incomplete, the blood-brain barrier is more permeable, neurons are more predisposed to apoptosis, and oligodendrocytes are also more vulnerable to oxidative injury and death than in mature brain. Similarly, the immune response of preterm and term infants is immature, with an exaggerated TLR4-mediated IL-6 increase and decreased TNF-α when compared with older children or adults (25,26). The time course of the neonatal response to HI-induced brain injury also differs from the adult response. For this reason, we focused on the time point 24-h after injury to identify immediate consequences of HI-induced injury on gene expression and to identify whether repeated high-dose rEpo treatment (two daily doses) altered these early response mechanisms.

We found remarkably few effects of high-dose rEpo on gene expression in mouse hippocampus in the absence of brain injury. This may reflect the small proportion of circulating rEpo that crosses the blood-brain barrier in the absence of brain injury (27). This result corroborates our previous finding that Epo concentrations in homogenized injured brain were double that of the uninjured contralateral hemisphere (28). In contrast to the minor changes in gene expression caused by high-dose rEpo in sham controls, the effects of rEpo
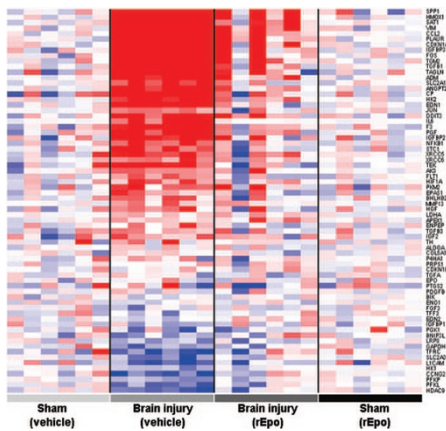
Figure 3. Expression of *C2 Hypoxia Review* gene set. A composite heat map with rows representing specific genes contained in the *C2 Hypoxia Review* gene set and each column representing a distinct sample that are arranged by treatment group labeled across the bottom.
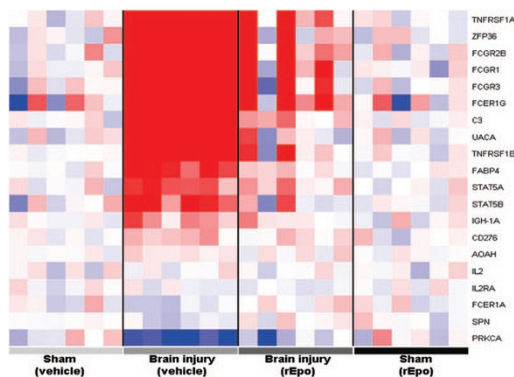

Figure 4. Expression of *BP Regulation of Inflammatory Response* gene set. A composite heat map comprised of rows representing genes contained in the *BP Regulation of Inflammatory Response* gene set and columns representing individual samples that are organized by treatment group, labeled along the bottom.

in the context of brain injury were large. Figure 2 shows that most genes up- or down-regulated by brain injury were largely normalized by rEpo treatment (92%). Although individual variations between animals in each group, and by sex were identified, the overall effects of rEpo were robust. These effects are expected because we intentionally compared the visibly brain-injured vehicle-treated mice to the visibly protected rEpo-treated mice in an effort to focus precisely on the genes underlying rEpo-mediated neuroprotection. This study was not designed to demonstrate the neuroprotective effectiveness of rEpo but, rather, to profile the patterns of gene expression associated with a neuroprotection phenotype.

Our most striking result was how profound the inflammatory response was to brain injury at 24 h. Gene sets related to all inflammatory cell lineages (monocytes, T cells, neutrophils, lymphocytes) were up-regulated by HI, as were mediators of inflammation; cytokines, chemokines, and complement. The frequency of changes in transporter gene expression was unexpected. Also surprising was the lack of angiopoietic gene expression in the rEpo-treated animals. This may indicate that the angiopoietic effects of rEpo after brain injury are delayed beyond 24 h and are thus less important for mitigating early injury than previously suspected.

Of interest, male animals showed more changes in gene expression because of injury than did females. Males also showed a more robust response to rEpo treatment. Clinically, we have not observed a difference in severity of brain injury in either treated or untreated animals short term, but we have not yet examined possible differences in long-term outcomes. Interpretation of the current dataset is limited by the small sample size.

Brain-injured animals treated with saline showed a 2-fold increase in HIF-2α. These data are consistent with previous reports showing that endogenous brain Epo production by astrocytes is up-regulated by hypoxia (29). Interestingly, rEpo treatment decreased this up-regulation, suggesting a possible feedback mechanism. We identified an up-regulation of HIF-1α, IL-1β, IL-6, and *c-fos* in the HI animals compared with the sham animals. This finding is consistent with other reports of HI-induced effects (11,14,30,31). In addition, the observed up-regulation of iNOS (NOS2) and an iNOS transcription-activating element, NF-κB, in the HI animals compared with the shams confirms prior immunohistological data (32).

In contrast, we did not confirm an up-regulation of neuronal pentraxin 1 (NP1) in the HI hippocampus, as has been reported by Hossain (33). This brain-specific protein is homologous to C-reactive and acute-phase proteins in the immune system and is thought to be important in excitatory synaptic remodeling. Our data showed an increase of NP1 gene expression in sham animals compared with HI animals. This discrepancy is likely due to differences in the details of the models used: age of the animals, timing of harvesting of RNA, and mechanism of injury.

In summary, we now confirm that repeated high-dose rEpo significantly alters gene expression to counter the effects of brain injury. It is important to continue investigation of rEpo as a neuroprotective agent and thereby provide additional insight into mechanisms of hypoxic-ischemic brain injury in neonatal mice.

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