Effects of neonatal stress and morphine on murine hippocampal gene expression

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Abstract

Critically ill preterm infants experience multiple stressors while hospitalized. Morphine is commonly prescribed to ameliorate their pain and stress. We hypothesized that neonatal stress will have a dose-dependent effect on hippocampal gene expression, and these effects will be altered by morphine treatment. Male C57BL/6 mice were exposed to 5 treatment conditions between postnatal day 5 and 9: 1) Control, 2) mild stress + saline, 3) mild stress + morphine, 4) severe stress + saline and 5) severe stress + morphine. Hippocampal RNA was extracted and analyzed using Affymetrix Mouse Gene 1.0 ST Arrays. Single gene analysis and gene set analysis were used to compare groups with validation by qPCR. Stress resulted in enrichment of genes sets related to fear response, oxygen carrying capacity and NMDA receptor synthesis. Morphine downregulated gene sets related to immune function. Stress plus morphine resulted in enrichment of mitochondrial electron transport gene sets, and down-regulation of gene sets related to brain development and growth. We conclude that neonatal stress alone influences hippocampal gene expression, morphine alters a subset of stress-related changes in gene expression and influences other gene sets. Stress plus morphine show interaction effects not present with either stimulus alone. These changes may alter neurodevelopment.

Introduction

The effects of stress on health and development may be either positive or negative. For example, short-term acute stress can boost the immune system, but prolonged inescapable stress can have deleterious effects on learning, development, the immune system, and may increase susceptibility to degenerative diseases (1-3). Preterm infants in the NICU are exposed to prolonged inescapable stress. On average, a 28 week gestation infant spends 10-12 weeks (approximately the last trimester of pregnancy) in a NICU. During this time of

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rapid brain development they are separated from their mothers, handled repeatedly, exposed to multiple painful procedures, and they may also be mechanically ventilated, gavage fed, and exposed to repeated periods of oxidative stress. Neurodevelopmental outcomes following extreme prematurity remain poor, with moderate to severe impairment occurring in close to 50% of extremely low birth weight infants (4). Autism, attention deficit disorder, and school failure also occur more frequently NICU survivors (5). While some degree of impairment might be inevitable, it is likely that the stress and treatments these infants undergo impact neurologic outcome. Improved understanding of these factors will provide the basis of better treatments and subsequent improvement in outcomes.

Many preterm infants receive opiates for sedation or analgesia during their NICU stay. Treatment occurs during a period of intense brain development, with brain weight tripling during the last trimester of pregnancy (6). Neuronal differentiation, migration, and synapse formation are active processes through term gestation, as is glial proliferation (7). Complex neural networks form in regionally specific ways (8, 9), controlled by families of netrins, ephrins, semaphorins, and Slits (10). In the rat, neonatal stress disrupts subsequent adult learning and maturation of the adrenal stress response (1-3). Morphine exposure may have additional consequences in the newborn, affecting both immune function and neurodevelopment (11-14). The combined effects of stress and morphine have not been well studied, yet are of vital importance since the at-risk period when critically ill infants are exposed to morphine is also a time during which they are experiencing inescapable stress (15).

Brain development in the third trimester of human gestation generally corresponds to the first two weeks of postnatal life in mice (16). Use of neonatal rodents to model preterm brain development is therefore reasonable, despite differences in brain complexity. We developed a rodent model of neonatal stress which simulates many of the experiences of a hospitalized preterm infant. We previously reported short-term hippocampal gliosis (17), and long term neurobehavioral effects of stress and morphine exposure in our mouse model of neonatal stress (18). To further investigate the mechanism by which these changes occur, we now hypothesize that repeated neonatal stress will have a dose-dependent effect on hippocampal gene expression, and that these effects will be altered by morphine treatment.

Materials and Methods

Animals

Adult wild-type C57BL/6 mice were purchased (Harlan) and housed under a 12 h light-dark cycle with free access to food and water. All animal procedures were approved by the Animal Care and Use Committee at the University of Washington.

Treatment Groups

Male mice were exposed to 5 treatment conditions between postnatal day (P)5 and P9 (n=3/group), with birth recorded as P1. Litters were culled to n=7 maximum per dam. Groups included: 1) Untreated controls (CC), 2) mild stress + saline (MSS), 3) mild stress + morphine (MSM), 4) severe stress + saline (SSS) and 5) severe stress + morphine (SSM). Only males were used so as to eliminate sex-related genetic variability. Untreated control animals underwent minimal handling on P5 and at euthanasia on P9. All animals were killed on P9. Groups 2-5 received s.c. 10 μL injections of either saline or morphine twice daily at 08:00 h and 15:30 h. The morphine (Baxter, USA) dose was 2 mg/kg in a 10 μL volume and based on the daily average litter weight. This dose produces circulating morphine levels that approximate the range measured in human preterm infants given standard intermittent i.v. morphine boluses, or continuous i.v. morphine infusion (19). Severely stressed pups (groups

4 and 5) were separated from the dam and isolated in individual containers within a veterinary warmer at 32°C from 08:00 h until 16:00 h, thus experiencing both maternal and littermate separation. Pups were gavage fed 50 - 150 μL of rodent milk substitute at 10:00, 12:00, and 14:00 h, using a 24 gauge animal feeding needle (Popper & Sons, New Hyde Park, NY). To simulate the oxidant stress of apnea, pups were exposed to hypoxia (100% nitrogen 1 min followed by hyperoxia (100% oxygen 5 min) twice daily (08:00 and 15:30 h). Mice were then returned to the dam with their concurrent unstressed littermates each evening and allowed to nurse overnight *ad lib*.

RNA methods

RNA from the right hippocampus was isolated (Cartagen Molecular Systems, San Carlos, CA). RNA quality was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc. 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, post-hybridization washings, and detection of the hybridized cRNAs using a streptavidin-coupled fluorescent dye. Hybridized Affymetrix arrays were scanned with an Affymetrix GeneChip® 3000 scanner. Image generation and feature extraction were performed using Affymetrix GCOS Software.

Statistical analysis and data normalization for Affymetrix Mouse Gene 1.0 ST Arrays

Raw microarray data were processed with Bioconductor (20). Probes were normalized with Robust Multi-Array (RMA) (21). From the normalized data, genes with significant evidence for differential expression were identified using the Bioconductor limma package (22). *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. *P*-values were adjusted for multiplicity with the Bioconductor package q-value (23), which allows for selecting statistically significant genes while controlling the estimated false discovery rate.

Single gene analysis

The experimental design, with the specific main effects and the interaction effects that we wished to examine are shown in Figure 1 and Table 1. Figure 1 shows the basis for our comparisons, and what specific questions they answer. We define a 1) stress-affected group of genes as the union of the mild and severe stress response genes (MSS-CC union with SSS-MSS) and 2) a morphine-affected group of genes as the union of the morphine response genes in the context of mild and severe stress (MSM-MSS union with SSMSSS), and 3) interaction effects of severe-stress with morphine as genes that react differently to morphine in the presence of severe stress than with mild stress.

Table 1 outlines the mathematical considerations we took into account when making group comparisons. For example, to answer the question "which genes increased by stress ($|\beta_1 + \beta_2| >> 1$) does morphine affect?" we look for genes that satisfy the equation: $\alpha_1 + \alpha_2 + \beta_1 + \beta_2 + \gamma_1 + \gamma_2 + \gamma_3 + \gamma_4 \sim 0$.

Gene Set Analysis (GSA)

To investigate categories of genes where the constituent genes show coordinated changes in expression over the experimental conditions, we used GSA, a type of biological category analysis (20, 24). GSA software is available as R code (24, 25) (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. The term "gene set" refers to genes that are grouped together based on function. For example, each gene ontology category (e.g. apoptosis) is a gene set. We used four gene set databases for GSA: Biological Process, Molecular Function, and Cellular Component from Gene Ontology (26), and the C2 gene set from the Molecular Signature Database (25) (http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html.

Validation of microarray data using fluorogenic 5' nuclease-based assay and quantitative RT-PCR (qPCR)

RNA from separate, additional animals were used for validation. Quantitation of specific mRNA levels were determined as previously described (27) using fluorogenic 5' nuclease-based assays on an ABI 7900 Sequence Detection System (28).

Results

Forty-seven genes were affected by mild stress, and 156 genes were affected by severe stress (up or down fold change > 1.3 fold and *p*-value < 0.05) including *Dnajb6* (*Hsp40* homolog), aminolevulinic acid synthase 2 (*Alas2*), and secreted phosphoprotein 1 (*Spp1*). Morphine had effects on 3 broad categories of genes: 1) morphine-responsive genes which were unaffected by stress (n=92); 2) morphine-responsive genes which were also stress-responsive (n=8); and 3) genes which responded only when both morphine and stress were present together, i.e. a morphine X stress interaction effect (n=104). Morphine-responsive genes were defined as those which changed expression more than 1.3-fold (up or down) with *p*<0.05 in either the mild stress (MSM-MSS) or the severe stress (SSM-SSS) contrasts. A subset of morphine-responsive genes are shown in Table 2. In comparison, a subset of the interaction-specific genes are shown in Table 3. Examples of how treatment conditions affect gene expression are illustrated in Figure 2 using qPCR. The relationship between genes affected by mild and severe stress are shown in the Venn diagram in Figure 3.

GSA allowed us to examine patterns of gene expression associated with each treatment group. Mild stress resulted in upregulation of many gene sets related to immune function including: IFNγ production, IL-1, 2 and 12 synthetic processes, defense response to gram-positive bacterium, regulation of T-helper cell differentiation, positive regulation of cell killing and leukocyte mediated cytotoxicity. Other upregulated gene sets included: positive regulation of systemic arterial blood pressure and glucocorticoid metabolic process. Down regulated gene sets included those related to DNA and cellular maintenance.

When animals undergoing severe stress were compared to controls, gene sets consistent with stress such as multicellular organismal response to stress and fear response were overexpressed. Unexpected, however, were the many upregulated gene sets related primarily to neurodevelopment and inflammation: dendrite morphogenesis, microtubule bundle formation, synaptic vessel exocytosis, cerebral cortex cell migration, axon regeneration, neurite development, *wnt* signaling pathway, ionotropic glutamate receptor signaling pathway, axon regeneration, nerve growth factor receptor signaling pathway, cell proliferation in forebrain, regulation of synapse structure and activity; IL-6 production, T cell signaling pathway, and regulation of cytokine production (GSA *p*-value < 0.001, false

discovery rate < 0.001). Severe stress downregulated gene sets related to glutamine family amino acid catabolic process, glutamate metabolic process, mismatch repair, nuclear mRNA splicing, phagocytosis, histone modification, DNA metabolic process, folic acid synthesis, and telomere maintenance. Supplementary Tables S1A and SB (**http://links.lww.com/PDR/XXX**) give more details of severe stress related gene changes.

When comparing animals exposed to mild stress plus morphine (MSM) with mildly stressed animals (MSS), there was down-regulation of many gene sets related to immune function, particularly T and B cell function, IL-1 and IL-12 synthesis. Overexpressed gene sets were not as thematic, but included glutamine and dopamine metabolism, hyperosmotic response, and growth hormone secretion. Supplementary Tables 2A and B (**http://links.lww.com/PDR/XXX**) give more complete lists of gene sets whose expression is modified by mild stress plus morphine. Note that it is not possible to determine the effects of morphine in the absence of stress, since handling the animals to give injections is a form of stress. Therefore, the comparison MSM versus MSS provides the best estimation of the morphine effect alone.

In contrast to mild stress plus morphine, severe stress plus morphine positively enriched gene sets were related in large part to mitochondrial electron transport, oxygen transport, ATP synthesis, ATP coupled electron transport, oxidative phosphorylation, cellular response to stress, regulation of dendrite morphogenesis, Notch signaling pathway, regulation of apoptosis, amyloid precursor metabolic process, and regulation of acute inflammatory response. Downregulated gene sets in this comparison included many gene sets involving neuronal development: regulation of axon extension, synaptic vesicle exocytosis, hippocampal development, cerebral cortex cell migration, dendrite development as well as several gene sets related to histone modification. Examples of these changes are shown in Figures 4 and 5; Figure 4 depicts the effects of combined severe stress and morphine on genes involved in microtubule bundle formation, dendrite development and regulation of axonogenesis, Figure 5 shows genes involved in aerobic respiration, cellular response to stress and ATP coupled electron transport. Supplementary Table S3,A and B (**http://links.lww.com/PDR/XXX**) give more complete lists of gene sets whose expression is modified by severe stress plus morphine.

We selected a subset of genes to validate by qPCR. Figure 6 shows the comparison of qPCR *vs*. microarray data. The correlation varies somewhat by condition, but overall the correlation is excellent.

Discussion

As survival of the smallest infants improves, optimizing their neurodevelopmental potential becomes paramount. While some degree of impairment following extreme prematurity might be inevitable, it is just as likely that over time, research will uncover pre and postnatal treatments to protect the developing brain and improve outcomes. Most preterm infants receive analgesics and/or sedatives during their NICU stay, sometimes for weeks (29). During this period of neuronal network development, factors that influence neuronal excitation and inhibition result in profound changes in the balance of excitatory and inhibitory pathways as well as in cell survival (30). Clearly, both stress and opiate-exposure have this potential. Further delineation of the individual and interactive effects of opiates with stress is of vital importance since the at-risk period when critically ill infants are exposed to morphine is concurrent with severe stress (15).

In this study, we show that repeated neonatal stress in mice has a dose-dependent effect on hippocampal gene expression, and that the interactions of stress with morphine are complex.

While morphine does down-regulate some stress-related changes in gene expression, this effect is not consistent across all stress-related genes. Depending on the gene in question and the degree of stress, treatment with morphine can have quite different and unpredictable effects. Figure 2 shows some examples of this: *Wnt* and *Spp1* expression are changed in the presence of morphine, but the direction of change depends on the level of stress. These changes in hippocampal gene expression may significantly impact neurodevelopment, and subsequently, behavior.

Mild stress tended to increase genes related to immune function, blood pressure, and glucocorticoid expression while decreasing those related to cell maintenance. Severe stress had less immune modulatory effects, but increased expression of gene sets having to do with neurodevelopment and inflammation, while down regulating gene sets related to cellular repair such as telomere maintenance, mismatch repair, DNA metabolic process, folic acid synthesis, nuclear mRNA splicing, and histone modification.

Morphine exposure had different effects on hippocampal gene expression in the presence of mild *vs*. severe stress. Treatment of mild stress with morphine increased oxygen transport, mitochondrial membrane maintenance and increased expression of specific gene sets related to neurodevelopment. Cholesterol, proteoglycan and steroid synthesis were downregulated. In contrast, treatment of severe stress with morphine markedly up-regulated expression in several gene sets regulating energy metabolism, oxidative phosphorylation and aerobic respiration. Thus it is apparent that the modulatory effects of morphine on hippocampal gene expression are dependent upon the level of neonatal stress. Of particular concern, there was marked down-regulation in gene sets involving RNA processing and neurodevelopmental processes. These data suggest a mechanism by which decreased brain growth and other deleterious effects of morphine might be mediated.

This study is limited in that we could not assess the isolated effects of morphine, since morphine injections are by themselves stressful, and we also did not differentiate between the stressful stimuli of maternal separation, pain, formula feeding by gavage, and hypoxia/hyperoxia in the severely stressed group. These stimuli were combined to simulate the experiences preterm infants are exposed to. To assess isolated morphine effects we compared mildly-stressed morphine-treated animals with mildly-stressed animals. This comparison showed upregulation of estrogen metabolism, blood volume regulation, growth hormone synthesis, and glutamine metabolism whereas immune function, urea cycle metabolism and cell movement processes (microtubule polymerization) were down regulated.

Our data are consistent with previous work showing differential expression of *Dnajb1* (*Hsp40*), and glutamate receptor ionotropic kainate 2 (*Grik2*) in the C57BL6 striatum after morphine treatment (31). Our results in regards to Neuropeptide Y (NPY) are also similar to a recent study showing 4 day morphine administration to mice caused an up-regulation of hypothalamic NPY gene expression (32). In addition, we found an up-regulation of galanin gene expression after morphine administration that is consistent with previous work demonstrating an increase in galanin gene expression in mouse locus ceruleus and ventral tegmental region tissues after chronic morphine treatment (33). Erabi *et al* showed that neonatal stress (isolation) resulted in long term changes the *IGF-IR* and *IGFBP-2* in the hippocampus in response to adulthood restraint stress, suggesting that epigenetic modifications occurred at the time of neonatal isolation (34). In our study, we found, but did not validate, changes in gene sets that relate to histone modification, which is a primary mechanism of epigenetic modification.

This study has provided a great deal of information about the neonatal hippocampal response to clinically relevant neonatal conditions: minor stress, severe stress including oxidative stress, and the interaction of morphine treatment with these conditions. We have validated differential expression of 16 genes of the 156 identified by microarray. We used RNA from a separate set of animals because we reasoned that if the findings were reproducible in these new animals, it not only validated the initial findings, *vis a vis* the validation of the technique, but also provided information regarding the reproducibility of the findings in animals treated with stress and/or morphine. Some of the differentially regulated genes are of uncertain importance, but others are likely to be of great importance and should be further studied. Many questions have been generated by this study. Why are genes and gene families related to energy metabolism upregulated by the combination of stress and morphine? Is this beneficial or deleterious? What is the mechanism by which morphine and stress alter neurogenesis, synaptogenesis, axon formation and neuronal differentiation? Are these effects long lasting? Are they dose-dependent? Is there a dose of morphine which does not impair these functions, or perhaps alternative sedatives that do not have the same developmental effects? Most importantly, the findings from this study must make us question whether the current practice in the NICU is best for our patients. While the treatment of unavoidable stress and pain in critically ill neonates is ethically mandated, the drugs we currently use may not, in fact, be effective in this population due to immaturity of the nervous system (35), and they may also cause harm to the developing brain by interrupting normal developmental functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

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GSA	Gene Set Analysis
MSM	Mild stress + morphine
MSS	Mild stress + saline
NPY	Neuropeptide Y
SSM	Severe stress + morphine
SSS	Severe stress + saline
P	Postnatal day
qPCR	Quantitative PCR
CC	Untreated controls

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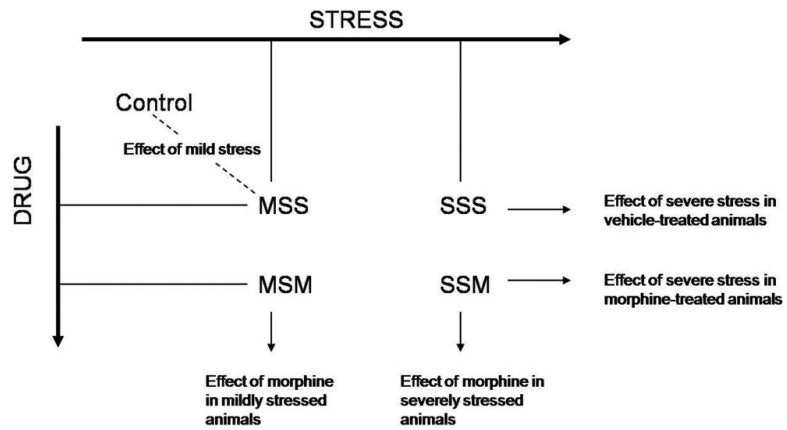
Figure 1. Stress morphine comparison groups
These comparisons allow us to answer the following questions: 1) What is the effect of mild stress? Answer: MSS-CC; 2) Is there a dose-dependent effect of stress? Answer: SSS-MSS; 3) How does severe stress affect morphine-treated animals? Answer: SSM-MSM; 4) How does morphine affect mildly stressed animals? Answer: MSM-MSS; 5) What does morphine do to severely stressed animals? Answer: SSM-SSS; 6) Does morphine act different in severely stressed animals? Answer: Stress X Morphine interaction
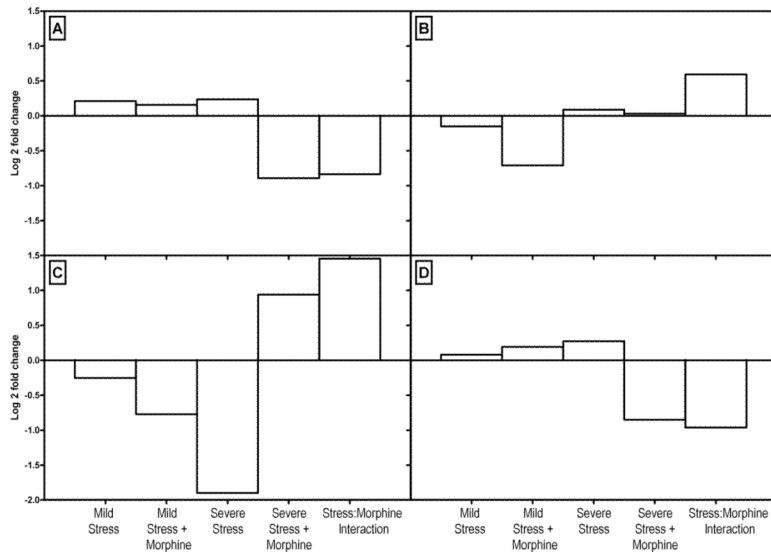
Figure 2. qPCR of selected hippocampal genes by treatment condition
qPCR results are normalized to GAPDH. The X axis shows the conditions: mild stress, mild stress + morphine, severe stress, severe stress + morphine and interaction effects of stress. The Y axis shows the log 2 fold change in gene expression. Expression of *Nav1* (Panel A), *Wnt2* (Panel B), *Spp1* (Panel C), and *Reln* (Panel D) differ by treatment group, demonstrating that the effect of morphine is not necessarily predictable.
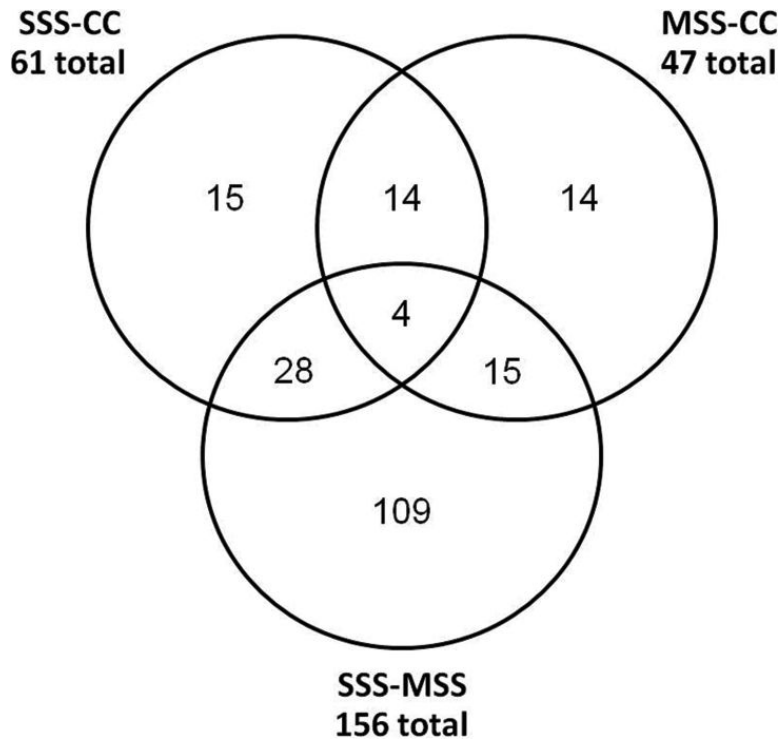
Figure 3. Venn diagram
This figure shows the relationship between genes affected by severe stress (SSS-CC), mild stress (MSS-CC) and the difference between mild and severe stress (SSS-MSS). Criteria for inclusion include |fold| > 1.3, $p < 0.05$.
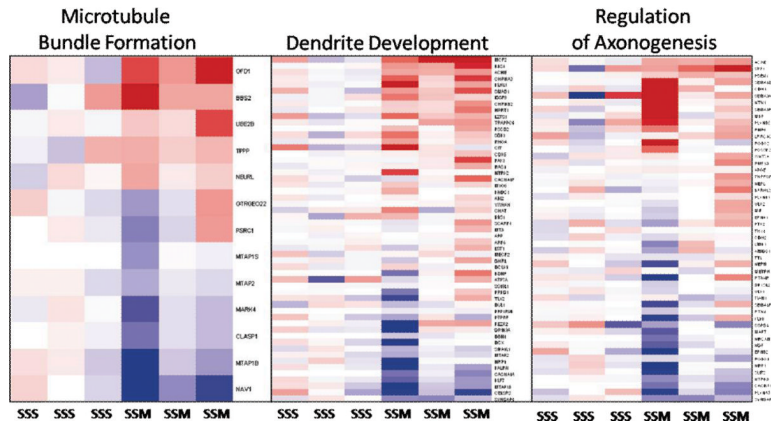
Figure 4. Neonatal stress and morphine treatment effects on selected neuronal activities
These composite heat maps show examples of three biological process gene sets related to neuronal development which differ by treatment group (GSA *p*-value< 0.0001). Each row represents a specific gene in the gene set, and each column shows the pattern of RNA expression from an individual animal, arranged by treatment group labeled across the bottom. Individual animal variability can be seen, as well as group differences. 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 control animals. Since the number of genes in the gene sets precludes adequate visibility, supplementary table S4 (**http://links.lww.com/PDR/XXX**) is provided listing the gene names in the same order as they appear in the heatmaps (from top to bottom).
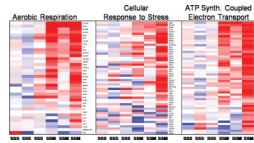
Figure 5. Neonatal stress and morphine treatment effects on cellular metabolism gene sets
Composite heat maps from severely stressed animals compared to severely stressed animals treated with morphine. Highlighted are gene sets related to energy metabolism. While there is some variability from animal to animal (columns), and by individual gene (rows), the treatment of stress with morphine clearly increased gene expression in many of the genes in each family relating to aerobic respiration, cellular response to stress, and ATP synthesis coupled electron transport (GSA *p*-value < 0.0001). Since the number of genes in the gene sets precludes adequate visibility, supplementary table S5 (**http://links.lww.com/PDR/XXX**) is provided listing the gene names in the same order as they appear in the heatmaps (top to bottom).
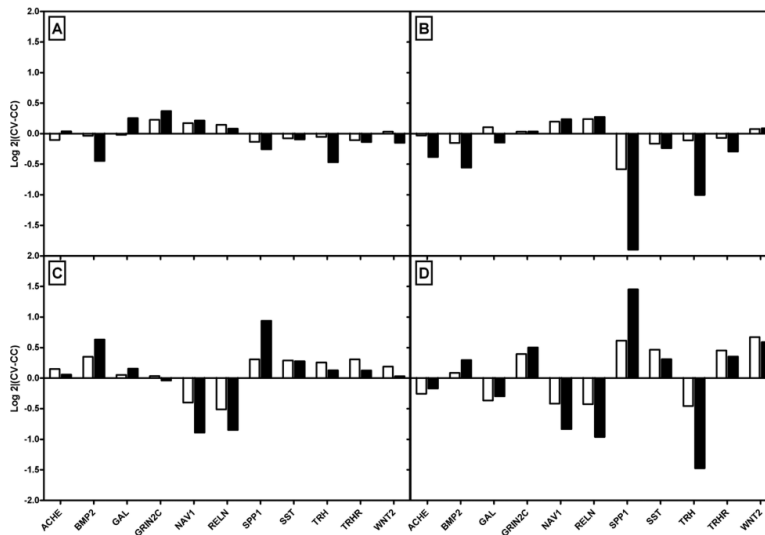
Figure 6. Validation of Microarray Results by qPCR
Microarray results were validated in 16 genes in all comparison conditions. A subset of 11 genes and conditions are shown in this figure. Panel A shows effects of mild stress; Panel B the effect of severe stress; Panel C shows effects of severe stress combined with morphine, and Panel D shows the stress: morphine interaction effects. Microarray results are shown in clear bars, and qPCR results in black for 11 genes. Gene names are listed on the X-axis. The Y axis shows the log 2 fold change in gene expression.

Table 1

DRUG	STRESS		
	no	*Weight + injections*	*Weight + injections + separation + hypoxia/hyperoxia*
no	μ	$\mu + \beta_1$	$\mu + \beta_1 + \beta_2$
vehicle	$\mu + \alpha_1$	$\mu + \alpha_1 + \beta_1 + \gamma_1$	$\mu + \alpha_1 + \beta_1 + \gamma_1 + \beta_2 + \gamma_3$
vehicle + morphine	$\mu + \alpha_1 + \alpha_2$	$\mu + \alpha_1 + \beta_1 + \gamma_1 + \alpha_2 + \gamma_2$	$\mu + \alpha_1 + \beta_1 + \gamma_1 + \alpha_2 + \gamma_2 + \beta_2 + \gamma_3 + \gamma_4$

α_1 = effect of vehicle

α_2 = effect of morphine

β_1 = effect of mild stress

β_2 = effect of severe stress

γ_1 = interaction effect of vehicle, α_1, with mild stress, β_1, probably zero

γ_2 = interaction effect of morphine, α_2, with $\beta_1 + \gamma_1$, probably negligible

γ_3 = interaction effect of $\alpha_1 + \gamma_1$ with severe stress, β_2

γ_4 = interaction effect of $\alpha_2 + \gamma_2$ and $\beta_2 + \gamma_3$

Table 2

Selected Morphine-responsive genes[*], unaffected by stress (92 genes)

Gene Symbol	Gene Description	MSM-MSS (log₂FC)[**]	MSM-MSS p-value[†]	SSM-SSS (log₂FC)[**]	SSM-SSS p-value[†]
Accn4	amiloride-sensitive cation channel 4	0.400	0.114	**0.605**	**0.024**
Aqp1	aquaporin 1	**1.187**	**0.030**	0.260	0.600
Avpr1a	arginine vasopressin receptor 1A	**-0.704**	**0.024**	-0.160	0.570
Bex4	brain expressed gene 4	0.015	0.901	**0.389**	**0.006**
Carhspl	calcium regulated heat stable protein 1	0.221	0.300	**0.477**	**0.037**
Cdh22	cadherin 22	0.026	0.861	**0.405**	**0.016**
Cldn2	claudin 2	**0.744**	**0.029**	0.245	0.430
Ctnnal1	catenin (cadherin associated protein), alpha-like 1	**0.415**	**0.021**	0.137	0.396
Cux2	cut-like homeobox 2	**0.653**	**0.013**	0.007	0.977
Dlx5	distal-less homeobox 5	0.088	0.752	**0.614**	**0.044**
Dock10	dedicator of cytokinesis 10	0.122	0.445	**0.410**	**0.027**
Gabra4	gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 4	0.101	0.385	**0.392**	**0.004**
Gad2	glutamic acid decarboxylase 2	0.231	0.424	**0.609**	**0.0495**
Gal	galanin	**0.408**	**0.033**	0.074	0.669
Gh	growth hormone	-0.189	0.514	**-0.854**	**0.010**
Glra2	glycine receptor, alpha 2 subunit	0.171	0.414	**0.441**	**0.049**
Mpeg1	macrophage expressed gene 1	0.017	0.870	**0.384**	**0.003**
Npy	neuropeptide Y	0.305	0.052	**0.539**	**0.002**
Reln	reelin	-0.097	0.532	**-0.491**	**0.007**
Rgs6	regulator of G-protein signaling 6	**0.439**	**0.010**	0.066	0.657
Tac1	tachykinin 1	0.083	0.860	**1.374**	**0.012**
Trh	thyrotropin releasing hormone	**0.700**	**0.029**	0.275	0.350

Columns 1 and 2 list Gene symbols and gene descriptions. Columns 3 [MSM-MSS (log2FC)] and 5 [SSM-SSS (log2FC)] provide the log2 transformed change in expression caused by mild-stress-morphine (MSM) treatment relative to the mild-stress-saline (MSS) treatment, and the severe-stress-morphine (SSM) treatment relative to severe-stress-saline (SSS) treatment respectively. Columns 4 (MSM-MSS p-value) and 6 (SSM-SSS p-value) list the p-values associated with the aforementioned contrasts.

[*] morphine responsive genes are defined as changing >1.3-fold (up or down) with p<0.05 in either the MSM-MSS or the SSM-SSS contrast

[**] fold change is provided as log2, positive numbers indicate gene was expressed at higher level in MSM vs. MSS or SSM vs. SSS respectively, whereas negative numbers indicate the opposite, bold print indicates a > 1.3-fold absolute change

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[†] bold print indicates p<0.05.

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Table 3

Selected genes responding only when Stress and Morphine are present together (104 genes total).

Gene Symbol	Gene Description	Stress × Morphine Interaction (log2FC)[*]	Stress × Morphine Interaction p-Value
C1ql3	C1q-like 3	0.497	0.038
Calbl	calbindin-28K dehydrogenase/reductase (SDR family)	0.557	0.011
Dhrs7b	member 7B	0.396	0.011
Dnahc3	dynein, axonemal, heavy chain 3	-0.440	0.013
Dynclhl	dynein cytoplasmic 1 heavy chain 1	-0.385	0.005
Ephx2	epoxide hydrolase 2, cytoplasmic	0.384	0.004
Fbnl	fibrillin 1	-0.412	0.013
Hs6st2	heparan sulfate 6-O-sulfotransferase 2	0.4345	0.012
Htr3a	5-hydroxytryptamine (serotonin) receptor 3A low density lipoprotein receptor-related	0.591	0.018
Lrp1	protein 1 malic enzyme 3, NADP(+)-dependent,	-0.445	0.012
Me3	mitochondrial	0.401	0.035
Myo19	myosin XIX	-0.392	0.001
Nav1	neuron navigator 1	-0.385	0.015
Pknox1	Pbx/knotted 1 homeobox	0.434	0.001
Rab11b	RAB11B, member RAS oncogene family ribonucleotide reductase M2 B (TP53 inducible)	0.392	0.019
Rrm2b		0.384	0.010
Sst	somatostatin	0.497	0.006
Syt5	synaptotagmin V	0.379	0.014
Trhr	thyrotropin releasing hormone receptor	0.487	0.046
Ttll11	tubulin tyrosine ligase-like family, member 11 thioredoxin domain containing 4	0.441	0.008
Txndc4	(endoplasmic reticulum)	0.381	0.019
Zfp239	zinc finger protein 239	0.380	0.009

[*] fold change is provided as $\log_2$, positive numbers indicate that the Stress X Morphine interaction effect increased gene expression, whereas a negative number indicates the opposite.