

Quantitative Proteomics in Combination with Immunomics Reveals Morphine-Produced Immunosuppressive Effects at the Protein and Cellular Levels in Non-human Primates

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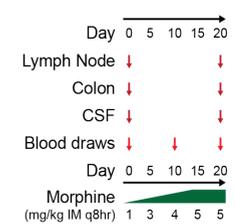
Overview

- Goal:** To identify the systemic changes induced by morphine in two primate species: African green monkeys (AGM) and pigtailed macaques (PT).
- Findings:** Decreased levels of (Ki-67+) T cell activation in both species; only minimal changes in overall T cell counts, neutrophil counts, and NK cells counts.
- Morphine-induced suppressive effect in lymph nodes, with decreased abundance of protein mediators involved in energy metabolism, signaling, and maintenance of cell structure.

Introduction

- With 20 million heroin abusers estimated worldwide and countless others who abuse prescription opioids, drug addiction remains a large public health problem. Addicted individuals are susceptible to a broad array of infectious complications and, in the US alone, drug abuse contributes directly to a third of all HIV infections.
- Opioids induce effects across a multitude of biological systems *in vivo* and these are not easily categorized into meaningful patterns using standard experimental approaches.
- The effects of *in vitro* experiments measure only the direct effects of these drugs without taking into account indirect and secondary pathways that may propagate opioid-associated impacts within a biological system.
- Objective: To obtain a better understanding of the underlying mechanisms of drug-induced pathology may allow more informed application of opioid replacement therapy as well as the development of additional novel therapeutic interventions.

Experimental



Analyses		
Cells	Cytokines	Proteomics
+	+	+
+	+	+
+	+	+

Design

- Morphine administered to animals, starting at 1 mg/kg intramuscularly every 8 h and titrated upwards to a maximum dose of 5 mg/kg by day 20.
- Animals sampled across a variety of biological compartments.
- Peripheral blood sampled at days 0, 10, and 20, while iliac lymph node biopsies, colonic mucosal biopsies, and cerebrospinal fluid samples obtained at days 0 and 20.
- Tissues and fluids subjected to cell phenotypic, cytokine, and proteomic analyses.

Note: All animals and *in vitro* procedures were performed using standard protocols and according to guidelines approved by the University of Washington Environmental Health and Safety Committee, the Occupational Health Administration, the Primate Center Research Committee, and the Institutional Animal Care and Use Committee.

Methods

Flow Cytometry

- Performed on a 4-laser LSR-II flow cytometer (BD Biosciences).
- Peripheral blood mononuclear cells isolated by density centrifugation using Histopaque=1077 (Sigma Aldrich), and stained using a variety of phenotyping panels.
- Tissue samples were mechanically disrupted, digested with collagenase type II, strained, and washed for mononuclear cells.

Sample preparation for proteomics

- Samples were denatured in 8 M urea, and quantified by BCA. Samples were reduced, diluted 10-fold, digested using trypsin, desalted using SPE, and then dried using Speed-Vac.
- Whole blood was collected in EDTA collection tubes, centrifuged, and the top 5 mL of the top plasma layer was collected, treated with EDTA-free Complete Mini Protease Inhibitor Cocktail (Roche), and stored at -20 °C. Albumin and IgG depletion was performed using the ProteoExtract Albumin/IgG removal kit (EMD Biosciences).

NanoRPLC-MS/MS

- RPLC system coupled to an LTQ-Orbitrap Velos using an electrospray ionization source.
- Operated in data-dependent mode with *m/z* 400-2000; 10 most abundant ions from MS selected for MS/MS using normalized collision energy setting of 35%.

Data analysis

- Spectral data searched using SEQUEST and the Ensembl protein list (obtained from InParanoid) containing 21,905 nonredundant protein sequences. Neither primates have a fully sequenced genome and the Rhesus macaque is the closest relative with a fully sequenced genome.
- High resolution LC-MS features deconvoluted using Decon2Ls and aligned to a reference peptide database using VIPER; peptide alignments further refined with mass error < 2 ppm and FDR < 5%.

Statistical analysis

- Outlier datasets identified using a peptide-centric approach that considers 5 statistical metrics to generate a robust Mahalanobis distance score¹. Three datasets resulted in extreme peptide abundance distributions ($P < 1E-03$), and were removed from the analysis.
- Relative peptide abundances were transformed, and a model-based statistical approach imputed missing peptide abundance values.
- Protein-level ANOVA identified statistical changes, and Benjamini-Hochberg correction was applied to *P*-values.

Results

Proteomic variation Inter-individual / inter-species

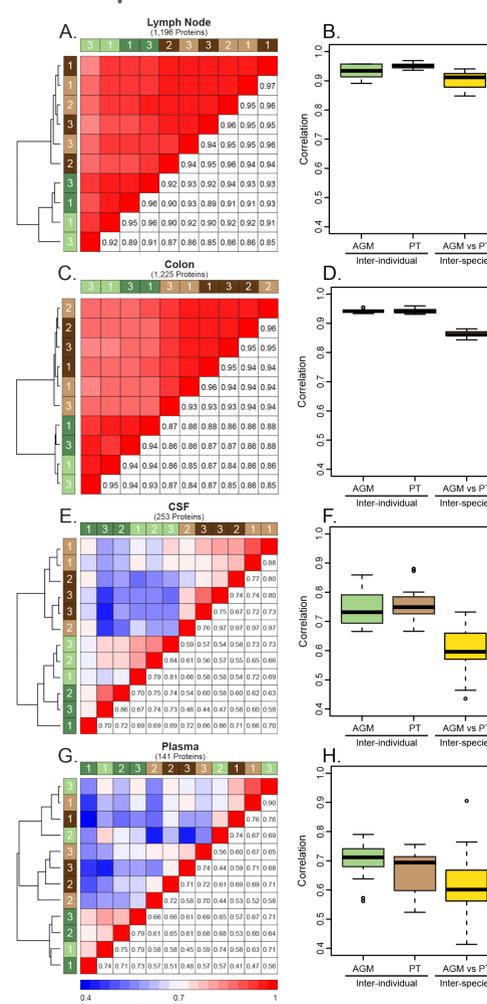


Figure 1. Proteomic variation across 132 high-resolution LC-MS were correlated to measure inter-individual and inter-species variation based on relative protein abundances between lymph node (A-B), colon (C-D), CSF (E-F), and plasma (G-H). The number of proteins considered in each analysis is represented in parentheses below the compartment title. Green bars indicate AGMs and brown bars represent PTs, with light and dark indicating pre- and post-morphine, respectively.

Cellular variation in lymph node - Inter-individual / inter-species

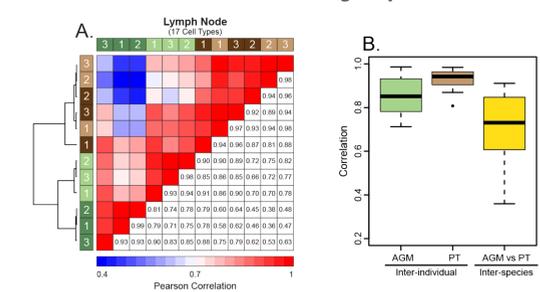


Figure 2. Similar analysis to Figure 1, except using cell counts from the lymph node as opposed to proteins. The number of cell types used in the analysis is above the heatmap in parentheses.

Activated T-cells in the lymph node

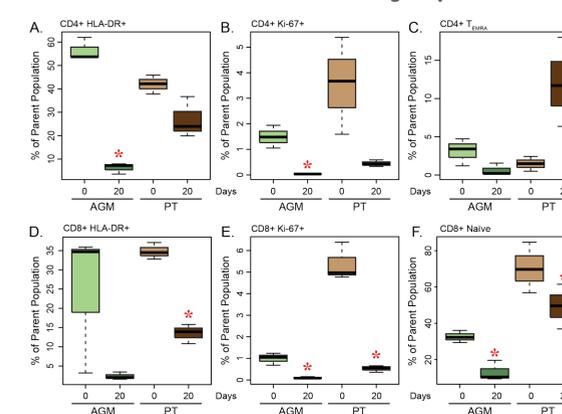


Figure 3. Boxplots representing the percent distribution of cell types measured in the lymph node.

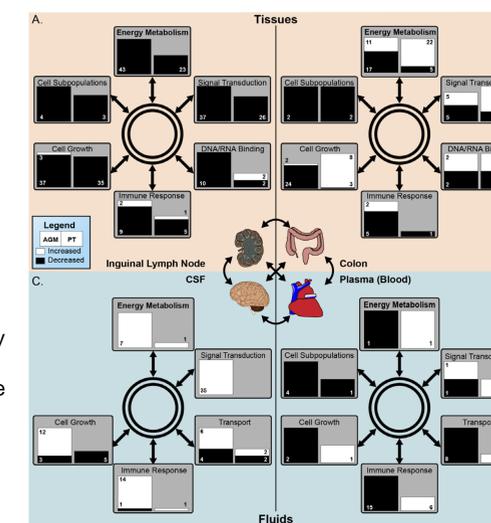
* indicates a significant change ($P < 0.05$) in cell count during morphine administration as determined by a paired t-test.

Table 1. Number & Percent of significantly expressed proteins.

Compartment	Number
Lymph node	422 (35%)
Colon	215 (18%)
CSF	113 (45%)
Plasma	56 (40%)

Integrated omics model schema of morphine immunosuppressive effects in NHPs

Figure 4. Samples were divided between tissues (A and B) and fluids (C and D). The left and right sides of the colored boxes indicated significant changes in AGMs or PTs, respectively. The size of the boxes are scaled to represent the number of proteins significantly altered within the functional category and numbers indicate the number of proteins whose abundance is significantly changed or the number of cellular subpopulations significantly altered in the case of "Cell Subpopulations". White represents a significant increase and black a significant decrease.



Conclusions

- Morphine produces an immunosuppressive effect in the lymph node (LN) of both NHPs, paralleled by decreases in activation levels of T cells in the peripheral blood of AGMs and PTs.
- Findings across gut mucosa, CSF, and plasma were inconsistent between species, suggesting that protein changes that are highly tissue- and species-specific accompany the response to morphine *in vivo*.
- Substantial decreases in energy metabolism proteins were concurrently measured in the LN, and may contribute to the observed decrease in T-cell activation.
- A number of these proteins have been previously reported to be decreased by morphine in other animal models, including MDH2 and TPI2.
- These findings highlight the need for alternative analgesics in the clinical setting that do not produce the same peripheral morphine-associated side effects, including immunosuppression and gastrointestinal dysfunction³.

Acknowledgements

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