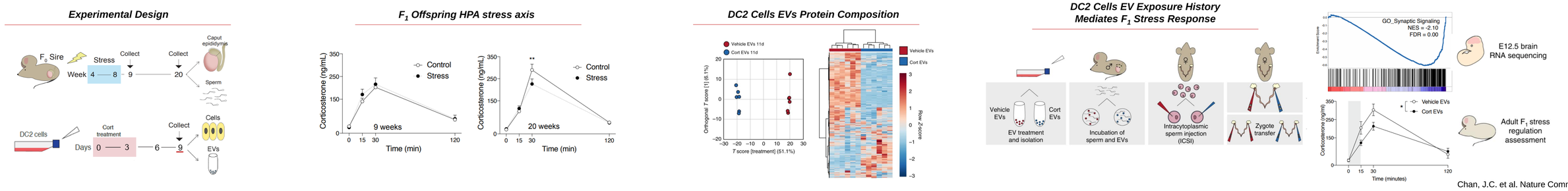
Stress-mediated cellular mechanisms of allostasis regulate mitochondrial bioenergetics following stress

Nickole Kanyuch[1,2], Jennifer Chan[2], Christopher Morgan[2], Ruth Marx-Rattner[2], Nagendra Yadava[3], & Tracy L. Bale[2]

[1]Program in Neuroscience, [2]Department of Pharmacology, CERCH, [3]Department of Anesthesiology, STAR, University of Maryland School of Medicine

Molecular mechanisms of intergenerational stress transmission mediated by caput epididymis EVs



Chan, J.C. et al. Nature Commun. 2020

Background

Paternal life experiences are associated with lasting effects that alter reproductive and embryo neurodevelopmental outcomes. Sperm maturation depends on signals such as extracellular vesicles (EV) from epididymal epithelial cells (EEC). Our previous work demonstrates that chronic stress changes EV signal, causally influencing post-conception neurodevelopment. However, the molecular mechanisms reprogramming EECs in response to stress is unclear. As a primary regulator of the stress response and allostasis, the glucocorticoid receptor (GR) presents a key target for investigation.

Chan, J.C. et al. Nature *Commun.* 2020

Hypothesis:
We hypothesize stress-dependent EEC programming involves GR and influences mitochondrial function.

Methods

Animals: GR^{flox} (B6.129S6-*Nr3c1*$^{tm2.1Jmy}$/J) and RiboTag (B6N.129-*Rpl22*$^{tm1.1Psam}$/J) mice were crossed with 129S1/SvImJ females for minimally 3 generations. Lcn5-Cre male mice on a C57Bl/6J background were bred to double heterozygous GR^{flox}; RiboTag 129 females to generate experimental animals. ***Chronic Variable Stress (CVS):*** Postnatal (PN) day 28, pair-housed males were exposed to one stressor each day for 28 days (PN28-56). Order or stressors was randomized each week. Stressors included the following: 36 h constant light, 1 h exposure to predator odor (1:5000 2,4,5 trimethylthiazole (Acros Organics) or 1:2000 phenethylamine (Sigma)), 15 min restraint, novel object overnight, multiple cage changes, 100 dB white noise overnight, and saturated bedding overnight. ***HPA axis assessment:*** Adult mice were tested 2-5 h after lights on by collecting 10 uL tail blood at onset and after completion of a 15 min restraint as well as at 15 and 115 min after the end of restraint (30 and 120 min times). 125I-corticosterone immunoassay (MP Biomedicals) determined corticosterone levels.

RiboTag immunoprecipitation: Whole caput epididymides were dounce homogenized in supplemented homogenization buffer and incubated for 4h with HA antibody. Antibody-protein complexes were isolated with Dynabeads overnight and washed 3x with high salt buffer before processing for RNA-seq library prep.

DC2 EEC corticosterone treatment: Immortalized mouse distal caput epididymal epithelial (DC2) cells cultured to confluency for 72 hours were treated with a single 500 mg/mL treatment of corticosterone (Sigma) or ethanol vehicle at the time of media replacement. Media was changed after 72 hours to washout treatment. Media was changed every 3 days until time of assay (day 9).

DC2 GR knockdown: Confluent DC2 cells were exposed to 18,000 transducing units of viral particles with 8 ug/mL hexadimethrine bromide overnight. Empty vector was used to control for the effects of transduction, non-mammalian shRNA control transduction Particles (Sigma) were used to control for the expression of shRNA, and MISSION shRNA Lentiviral Clone Oligo TRCN0000026223 was used to express shRNA to knockdown GR. Transduced cells were selected with puromycin. Surviving colonies were selected, passaged and cryopreserved until required for downstream assays.

Mito Stress Test (MST): Adherent DC2 cells were cultured and treated in XFe96 FluxPak plates (Agilent). On the day of the assay, media was changed to base media (Agilent) pH 7.4 supplemented with 1.0 mM sodium pyruvate, 4.0 mM glutamine, and 15.0 mM glucose. A Seahorse XFe96 cartridge was loaded with 1.5 µM oligomycin, 2 µM trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP), and 0.5 µM Rotenone/Antimycin A (Agilent) in base media. Media was replaced prior to the run.

Complex I Enzyme Activity: Activity was measured following the procedures outlined in the Complex I Enzyme Activity Microplate Assay Kit (Abcam) on an Infinite M1000 plate reader (Tecan) and analyzed using Magellan software (Tecan). Protein concentration for normalization quantified by Pierce BCA Protein Assay Kit (Thermo Scientific).

Transmission Electron Imaging (TEM): DC2 cultures were washed with PBS prior to overnight fixation with aldehyde-based fixative at 4°C. Samples were sectioned and imaged by the Electron Microscopy Core Imaging Facility at UMB. EM sample preparation instruments were purchased with funding from an NIH SIG grant (1S10RR26870-1) and a Department of Defense DURIP grant, Proposal No.70183-LSRIP.

DC2 CUT&RUN sequencing: The protocol was adopted from the Nature Protocols manuscript by Skene, Henikoff & Henikoff. DNA libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB) with the NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set I) (NEB). Individually barcoded libraries were pooled and paired-end sequencing was performed on an Illumina NextSeq 500.

Paternal GR reduction reverses EEC-driven paternal stress transmission and increases gene expression related to mitochondrial function

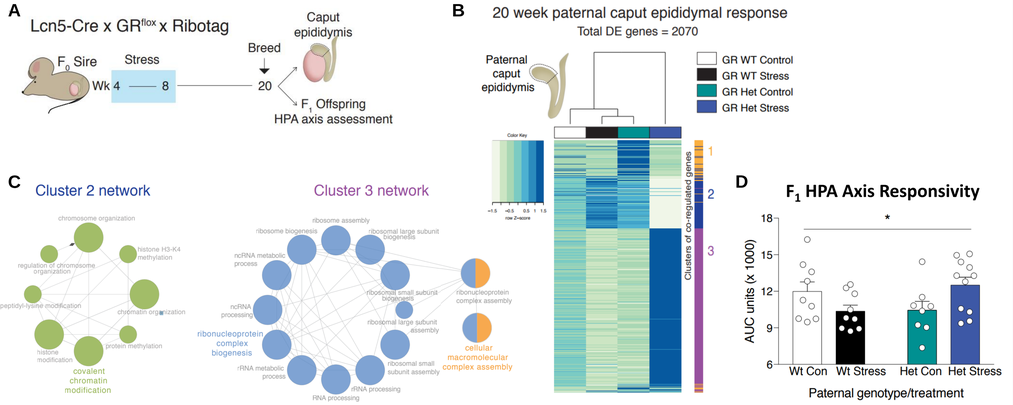


Figure 1. ***(A)*** Caput epididymal epithelial cell-targeted Lcn5-Cre x GR^{flox} x Ribotag (Rpl22) male mice were exposed to our established CVS stress protocol for four weeks and bred or sacrificed for epididymis collection at 20 weeks after a 12 week recovery period. ***(B)*** Heatmap of all differentially expressed genes from RNA-seq of paternal caput epididymal epithelial cells using Ribotag technology at 20 weeks. N= 4=6; adjusted P < 0.05. Hierarchical clustering of co-regulated genes depicted by color blocking on the right for functional annotation analysis. ***(C)*** Functional annotation analysis with biological processes gene ontology terms for cluster 2 and 3 revealing pertinent enriched pathways as determined by ClueGo. Connectivity indicated by edges between terms and statistical significance indicated by node size with the leading term (colored) determined by greatest significance. Number of associated groups per term indicated by node colors. ***(D)*** Two-way ANOVA of offspring HPA response to restraint area under the curve (AUC) demonstrates significant interaction for paternal treatment x genotype, interaction of paternal genotype x paternal treatment, N = 8-11; two-way ANOVA, *P < 0.05.

Stress-dependent changes in transcriptional repressor H3K27me3 associated with mitochondrial processes and increased mitochondria associated GR

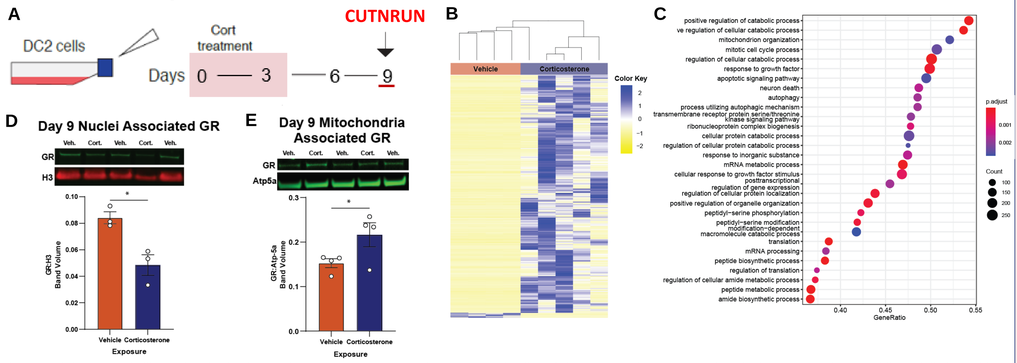


Figure 2. ***(A)*** Schematic illustrating the *in vitro* corticosterone exposure and CUTNRUN timeline. ***(B)*** Heatmap of peaks or regions in the genome that are differentially bound by H3K27me3. Each column is a sample and each row represents a peak. FDR <0.05, N=4-5. ***(C)*** Gene set enrichment analysis of all genes aligning with peaks bound by H3K27me3. Each row represents a pathway associated with genes. Color depicts adjusted p-value, size depicts the number of genes represented in the gene set. GeneRatio is calculated by dividing count by the total number of genes in the gene set. ***(D)*** Two-tailed t-test comparing GR protein in the nuclear fraction of DC2 cells exposed to corticosterone compared to vehicle. GR band volume normalized within lane to nuclear protein, H3, band volume. Representative western blot shown, *P <0.05, N=3. ***(E)*** Two-tailed t-test comparing GR protein in the mitochondrial fraction of DC2 cells exposed to corticosterone compared to vehicle. GR band volume normalized within lane to mitochondrial protein, ATP-5a, band volume. Representative western blot shown, *P <0.05, N=4.

Prior EEC stress exposure results in changes in allostatic set point via regulation of mitochondrial respiration dependent on GR expression

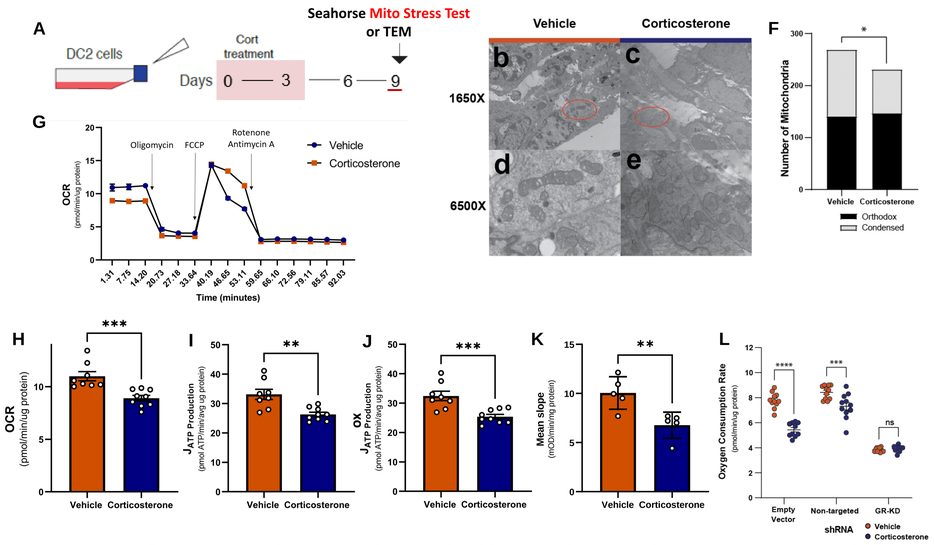


Figure 3. ***(A)*** Schematic illustrating the *in vitro* chronic stress model in EECs. ***(B) – (E)*** Representative day 9 vehicle or corticosterone treated DC2 cells from TEM. ***(B)*** 1650x magnification of vehicle treated DC2 cells. Red circle indicates representative mitochondria (mt) with condensed morphology, circled at at 6500x in ***(D)***. Condensed morphology characterized by dark color due to dense matrix and wide cristae. ***(C)*** 2700x magnification of corticosterone treated DC2 cells. Red circles indicate representative mt with orthodox morphology characterized by an expanded matrix with compact cristae. ***(E)*** 6500x magnification of corticosterone treated DC2 cells. Red circles indicate representative mt with orthodox morphology. ***(B) – (E)*** N = 231-269 mitochondria. ***(F)*** Comparison of mitochondrial ultrastructure between DC2 cells with prior corticosterone exposure and vehicle. Total number of mitochondria in each group with either orthodox or condensed ultrastructure denoted by color in the bar. Analyzed by chi square test. *P <0.05. (***G***) Representative plot of oxygen consumption rate (OCR) x time for Mito Stress Test injections. ***(H)*** Two-tailed t-test of exposure x basal mitochondrial OCR demonstrates decrease in OCR in day 9 corticosterone treated cells. ***(I)*** Two tailed t-test of exposure x total ATP production rate ($J_{ATPtotal}$) demonstrates decrease in $J_{ATPtotal}$ in day 9 cort treated cells,. ***(J)*** Two tailed t-test of exposure x oxidative ATP production rate (J_{ATPox}) demonstrates significant decrease in J_{ATPox} in day 9 corticosterone treated cells. ***(K)*** Two tailed t-test of exposure x complex I activity demonstrates decrease in complex I activity in day 9 cort treated cells, N = 5. ***(H) – (K)*** Error bars indicate SEM, **P <0.01, ***P or ****P <0.001, N = 8-9, ***(K)***, N=5 wells. ***(L)*** Two-way analysis of variance demonstrates decrease in OCR in DC2 cell with prior corticosterone treatment in both empty vector and non-targeted shRNA expressing control groups. No difference between exposure groups in GR-KD cells. Error bars indicate SEM, ***P or ****P <0.001, ns = not significant, N=10-12 wells.

Summary

- Chronic stress in male mice significantly blunts offspring HPA stress response which was reversed by selectively reducing GR in caput epididymal epithelial cells (EEC). Critically, this validated GR as a target mediating allostasis in the context of paternal intergenerational stress.
- Paternal stress produced marked, enduring and GR-dependent changes in EEC transcriptomics. Interestingly, a vast majority of these GR-responsive genes related to mitochondrial function. Similarly, H3K27me3 bound regions are associated with metabolic and mitochondrial processes.
- *In vitro* evaluation of mitochondrial function demonstrates decreased basal respiration and ATP production rate following corticosterone treatment. GR knockdown protects against stress-dependent changes in respiration. These data suggest a role for mitochondria in cellular regulation of allostasis.

Taken together, these data demonstrate the establishment of a new, enduring cellular allostatic set point in response to stress, and encourages further investigation regarding the related roles of GR in the regulation of allostasis in EECs and its influence on mitochondrial function and cellular baseline.

Support generously provided by NIEHS grant ES028202, NICHD grant HD097093, NIMH grants MH104184 and MH108286.