Glucocorticoid Receptor Phosphorylation and Neurological Disorders

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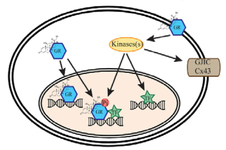
Introduction

- Premature birth, defined as birth prior to 37 weeks of gestation, occurs in 12% of pregnancies and is associated with significant morbidity and mortality[1].
- A significant risk for adverse clinical outcome is due to immature development of the lungs. Respiratory Distress Syndrome (RDS), due to a decrease in alveolar surfactant production and Bronchopulmonary Dysplasia (BPD) as a consequence of prolonged oxygen use and mechanical ventilation can lead to long term respiratory problems[2].
- Synthetic Glucocorticosteroids (sGC) are administered prenatally to pregnant mothers at risk for preterm birth between 23 and 36 gestation weeks to reduce the risks associated with RDS and BPD.
- While sGCs prove beneficial for the lung, several studies indicate that there is a correlation between long-term neurological defects in the infant and the clinical use of sGC prenatally[3].
- sGC act on the glucocorticoid receptor through both genomic (transcription regulation) and non-genomic (protein phosphorylation) pathways to induced a cellular response in both the lung and the brain.



- Site-specific phosphorylation of Glucocorticoid Receptor (GR) is associated with specific psychiatric disease states, including Major Depressive Disorder, Bipolar Disorder and Neuroticism (in women only), depending on the ratio of phosphorylation status of serine 211 and serine 226 in GRs[4].
- This study investigated the molecular and cellular consequences of premature activation of the glucocorticoid receptor in the developing brain in an animal model

Hypothesis

- sGC induced rapid phosphorylation of the glucocorticoid receptor at several sites. Changes in the ratio of phosphorylation on Serine 220 versus Serine 226 are associated with psychiatric abnormalities in adult patients.
- We hypothesize that phosphorylation of the receptor at serine 220 in neural stem cells leads to a change in their biological activity that leads to long-term neurological defects.

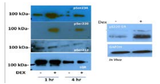
Methodology

- 2 different mice were used in this experiment: a wild type mouse and a knock-in mouse model where Serine 220 was replaced by alanine (S220A). Mice were provided by Dr. Cidlowski (NIH).
- Affymetrix Clariom S entire expressed genome gene chips, was used to identify the transcriptional changes in response to sGC.
- RNA was isolated from neural stem cells (MNSC) isolated from wild type and mutant mice, converted to cDNA using a RNA-to-cDNA kit exactly as outlined in manufacture's protocol (Fisher Scientific). Quantitative Polymerase Chain Reactions (qPCRs) were completed on specific genes of interest using Sybr Green (Sigmaaldrich) according to the manufacture's instructions.
- MNSC were cultured until P2, then seeded at $1.2x10^5$ cells/mL onto poly-d-lysine and laminin-coated coverslips. 10^{-7} M Dexamethasone (Dex) was added and cells were grown for 3 days.
- Immunohistochemical staining was performed with anti-Mouse Glial Fibrillar Acidic Protein (GFAP), anti-Rabbit Sox2, anti-Rat BromodeoxyUridine, anti-Rabbit Cy3 secondary, anti-Rat AF648, anti-Mouse Af488.
- Statistics were performed in Excel and samples were compared using T-test and significance assigned at 0.05

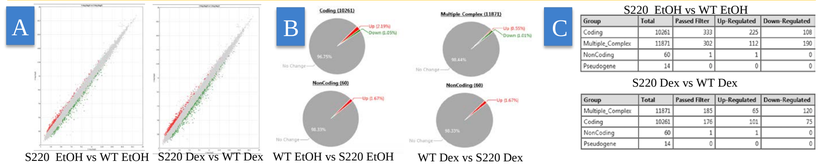
Results

1. The Glucocorticoid Receptor is Phosphorylated on Serine 220 and 226 in Neural Stem Cells

To determine the phosphorylation status of the GR in MNSC, P2 MNSC were exposed to Dex for 1 hour and western blots performed with antibodies to phosphorylated GR at S220 and S226. Both S220 and S226 are phosphorylated in MNSC and phosphorylation was increased by Dex exposure.



2. Schematic summary of microarray analyses



S220 EtOH vs WT EtOH

Group	Total	Passed Filter	Up-Regulated	Down-Regulated
Coding	18261	333	225	108
Multiple_Complex	11871	302	112	190
NonCoding	60	1	1	0
Pseudogene	14	0	0	0

S220 Dex vs WT Dex

Group	Total	Passed Filter	Up-Regulated	Down-Regulated
Multiple_Complex	11871	185	65	120
Coding	18261	176	101	75
NonCoding	60	1	1	0
Pseudogene	14	0	0	0

A. To identify transcripts that are altered by S220 phosphorylation, control and S220A cDNA was hybridized to a gene chip. Summary Scatter plot are shown with the $\log_2$ probe intensities of single genes that show a > 1.5 fold increase (red) versus a <1.5 decrease Green) in S220 knock-in verses a control basal expression (left) versus Dex induced transcript changes (right).
B. Pie chart summary showing the percent transcriptome changes detected in S220A mice compared to control mice.
C. Table summarizing the number and type of total genes assessed upregulated or downregulated.

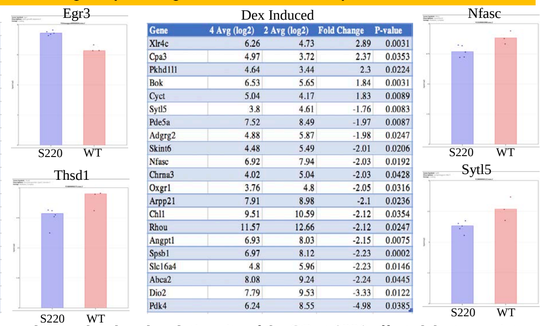
3. Summary of microarray analyses demonstrating sample changes in individual transcripts

Basal

Gene	3 Avg(log2)	1 Avg (log2)	Fold Change	P-value
Vmn2r35	5.89	4.53	2.56	0.0013
Mgat4c	5.76	4.54	2.33	0.0081
Csll3	4.96	3.81	2.22	0.0043
Egr3	7.44	6.28	2.24	0.0138
Vmn1r91	4.91	3.85	2.09	0.0129
Gm7697	6.03	4.97	2.09	0.0061
Zfp640	7.81	6.75	2.08	0.0101
Car3	11.46	10.58	1.84	0.0414
Thsd1	3.93	4.75	-1.77	0.0181
Tmem144	5.98	7.25	-2.41	0.0492
Elovl7	6.33	7.61	-2.43	0.0229
Bdkrb2	4.52	5.83	-2.47	0.0034
Sod3	3.89	5.21	-2.49	0.0239
Tceal1	3.29	4.63	-2.52	0.0438
Eml6	4.76	6.1	-2.53	0.0098
Slc6a15	5.2	6.54	-2.53	0.015
Sgc5	5.21	6.73	-2.87	0.0306
Abca1	9.24	10.84	-3.03	0.0406
Parvb	5.21	7.15	-3.83	0.022
Thy1	5.59	7.59	-3.98	0.0158
Ctsh	7.26	9.33	-4.21	0.0358

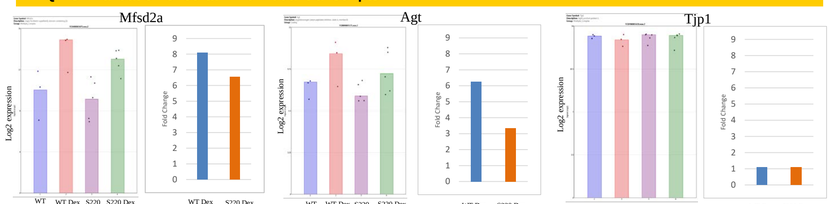
Dex Induced

Gene	4 Avg (log2)	2 Avg (log2)	Fold Change	P-value
Xlr4c	6.26	4.73	2.89	0.0031
Cpa3	4.97	3.72	2.37	0.0353
Pkhd1l1	4.64	3.44	2.3	0.0224
Bok	6.53	5.65	1.84	0.0031
Cyct	5.04	4.17	1.83	0.0089
Sytl5	3.8	4.61	-1.76	0.0083
Pde5a	7.52	8.49	-1.97	0.0087
Adgrg2	4.88	5.87	-1.98	0.0247
Skint6	4.48	5.49	-2.01	0.0206
Nfasc	6.92	7.94	-2.03	0.0192
Chrna3	4.02	5.04	-2.03	0.0428
Oxgr1	3.76	4.8	-2.05	0.0316
Arpp21	7.91	8.98	-2.1	0.0236
Chl1	9.51	10.59	-2.12	0.0354
Rhou	11.57	12.66	-2.12	0.0247
Angpt1	6.93	8.03	-2.15	0.0075
Spsb1	6.97	8.12	-2.23	0.0002
Slc16a4	4.8	5.96	-2.23	0.0146
Abca2	8.08	9.24	-2.24	0.0445
Dio2	7.79	9.53	-3.33	0.0122
Plk4	6.24	8.55	-4.98	0.0385



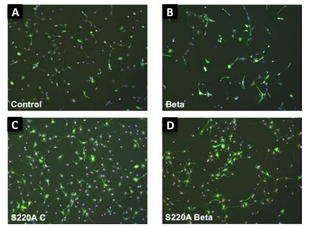
The Affymetrix Gene Chip measured how altering the phosphorylation site of the GR at S220 affected the transcriptome. 338 genes were upregulated and 298 were downregulated basally compared to controls. The basal levels of select target genes altered in the S220 MNSC are shown (left) with average sample signal for Egr3 and Thsd1. Select Dex induced target genes expression from the 167 upregulated and 195 downregulated genes are shown with sample signals for Nfasc and Sytl5 (right).

4. Q PCR Validation of certain individual transcripts



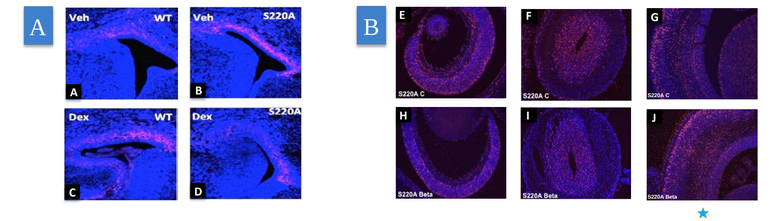
Select target genes were validated in an independent experiment (n=3-5). Sample signal sequences from Affymetrix Gene Array Chips (Log2) are shown on the left with their corresponding validation qPCR results on the right for Mfsd2a, Agt and Tjp1.

5. S220 Regulates Proliferation of Neural Stem Cells



To determine if the alterations in transcription observed in results 2-4 lead to biological changes, $1x10^4$ MNSC were seeded in a 96 well plate and exposed to Ethanol (ETOH) or sGC Betamethasone (Beta) for 24 hours. Cells were then stained with GFAP, a progenitor/glial marker (green) and Sox2, a stem cell marker (red). In controls, Beta decreases proliferation (B vs A). An increased number of cells is seen in control treated S220 cells (C) suggesting that basal s220 phosphorylation regulates stem cell proliferation. Beta decreases proliferation in S220 animals (D vs C). When comparing GFAP staining in S220 knock-in verses WT (C & D vs A & B), it appears that these cells are more mature as evidenced by the multipolar morphology of the cells.

6. Immunohistochemistry – In vivo effects of Beta GC treatment in knock in mice utilizing Brdu Staining



A. To determine if the changes observed in-vitro (5) are reproduced in-vivo, immunohistochemical staining of the Cerebral Cortex of S220 and wildtype mice exposed to Beta for 3 days is shown. Panels A shows increased Tbr2 staining (intermediate progenitor cell marker) in the S220 knock-in animal (B versus A, red). The opposite correlation is seen in Beta treated mice, the S220 knock-in inhibits progenitor cell proliferation (D versus C).
B. Top Panels (E-J) demonstrate BromodeoxyUridine (Brdu) staining in the eye (E), olfactory bulb (F), and Cerebral Cortex (G) of S220 knock-in animals of animals injected with Vehicle (E-G) or Beta (H to J) on E14.5 and collected on E17.5. In Panels H and I, fewer Brdu labeled cells are detected suggesting that Betamethasone decreased proliferation in S220 KI mice. Comparing Panels G and J, it appears that there are more Brdu labeled cells in the intermediate zone (star) which is where neurons are migrating.

Summary/Conclusion

- Substituting the serine at position 220 for an alanine residue alters the expression levels of a particular subset of genes without altering a majority of the genome. Some of the altered pathways include protein-protein interactions involved in cellular proliferation and G-coupled Protein Receptor involved in neurotransmission.
- In the cerebral cortex, basal S220 phosphorylation regulates progenitor cell proliferation as observed by changes in Tbr2, a progenitor marker and Brdu, a marker of cellular proliferation in-vitro and in-vivo. Betamethasone treatment alters the cellular response to S220 phosphorylation, it could be either stimulating proliferation of progenitor neural stem cells or slowing down neural stem cell migration to layer 2/3 of the cerebral cortex.
- Future studies will examine the role of select target genes identified in 1-4 in mediating the cellular responses of S220 phosphorylation. In addition, more detailed anatomical studies will identify the long-term consequences of S220A mutation.

References

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