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Additional Sex Combs-Like 2 (ASXL2): a Psychological Stress-related Gene in a Chicken Stress Model

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Additional Sex Combs-Like 2 (ASXL2): a Psychological Stress-related Gene in a Chicken Stress Model

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I. Abstract

Chronic stress is a problem not only in terms of its psychological effects but also due to the harmful physiological changes induced in the individual. Although chronic stress is recognized as detrimental and rampant throughout society, the stress system itself remains poorly understood in terms of comprehending which genes are involved in regulating and carrying out stress-induced signals. In a previous study, 57 candidate genes in the stress pathway had been identified based on the presence of stress-associated single nucleotide polymorphisms (SNPs) and differential expression from microarray data. The objective of this study was to investigate Additional Sex Combs-Like 2 (ASXL2), one of those 57 candidate genes, and to determine if it was part of the stress pathway through SNP identification and Real Time-Polymerase Chain Reaction (RT-PCR).

Utilizing sequencing data of ASXL2 from Pubmed as well as SNP locations generated from DNASTAR's SeqMan Ngen Assembly, SNPs were identified within the actual reference genome verifying the normal and mutant base pair for each SNP. After identifying SNPs of ASXL2 within the low stress line of Japanese quail, RT-PCR was performed in order to determine if mRNA expression for ASXL2 would change under acute and chronic stress. In order to perform RT-PCR, primers were designed and then tested utilizing gel electrophoresis in order to ensure optimization of the PCR product. $\Delta\Delta C_T$ methodology was then used to determine the veracity of the mean fold expression changes found for ASXL2 under acute and chronic stress.

As part of the experiment to determine the potential role of ASXL2 in the stress pathway, four SNPs were identified with high SNP rates in the low stress line of Japanese quail within ASXL2. After performing RT-PCR, it was found that ASXL2 was up regulated by 14% under

acute stress and down regulated by 24% under chronic stress in comparison to the acute control group and chronic control group respectively. P-values of 0.0013 and 0.0004 for acute and chronic stress respectively indicate that the data are statistically significant. β-Actin was set at a value of 1.0 as an internal control in order to determine mean fold expression changes for ASXL2 under differing stress conditions by using $\Delta\Delta C_T$ methodology.

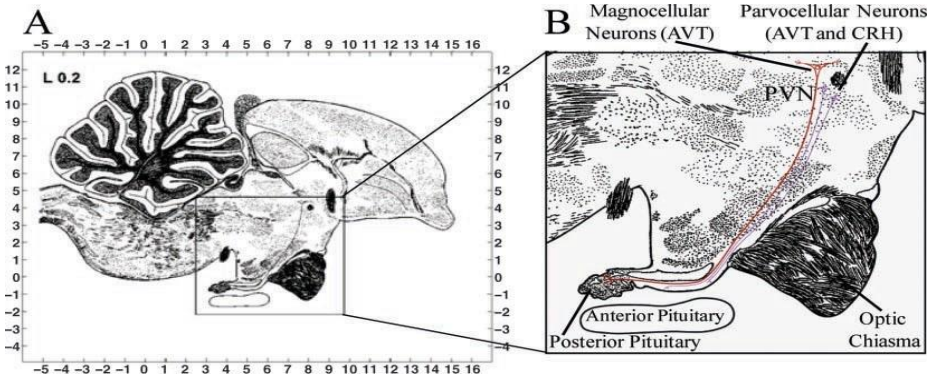
Based on the high SNP rate as well as the differential expression of ASXL2 under acute and chronic stress, ASXL2 has been directly implicated as a stress-related gene. Although ASXL2's exact role within the stress pathway is unknown, its interactions with trithorax and Polycomb group genes as an epigenetic modifier make it an interesting candidate for further research to determine its possible role in the epigenetic effects of acute and chronic stress.

II. Introduction

The effects of chronic stress are widespread throughout the body resulting in observable physiological changes as well as changes to the actual genome (Harris 2014; Quinlan *et al.* 2014). Hypertension, diabetes, immunological dysfunction and other physiological disorders are all associated with the presence of chronic stress (Harris 2015; Quinlan *et al.* 2014; Thompson and Haskins 2015; Williamson *et al.* 2014). Epigenetically, stress-induced DNA methylation of various genes causes dysregulation of certain physiological mechanisms leading to various psychiatric disorders (Hing *et al.* 2014; Tsankova *et al.* 2007). With such widespread negative implications for both physical and mental health, a better understanding of the genetic predisposition for chronic stress is needed.

The chicken stress model holds important implications for understanding the human stress pathway based on important similarities in terms of physiological stress response. Arginine vasopressin, a human neuropeptide secreted upon activation of the hypothalamic-pituitary-adrenal (HPA) axis for water retention purposes, is homologous to avian arginine vasotocin (AVT), differing in position 3 of the amino acid sequence (Selvam *et al.* 2013). Corticotropin-releasing hormone (CRH), secreted by the hypothalamus in reaction to stress, shares an identical 41-amino acid peptide sequence in both humans and chickens (Kuenzel *et al.* 2013). The avian and mammalian HPA axis both share similar receptors for neurohypophysial hormones as well as similar locations and expression levels of these receptors (Cornett *et al.* 2003). For example, one of the avian receptors for the neuropeptide AVT known as vasotocin receptor 4 and its homologous counterpart human V1b-vasopressin receptor are expressed majorly in the anterior pituitary and share functionality in mobilizing calcium and activating phosphatidylinositol breakdown (Cornett *et al.* 2003). Figure 1 below highlights the structures and pathway involved in the poultry stress response.

Figure 1- (A) Chicken brain: Boxed in view is the diencephalic region containing neuroendocrine components of the hypothalamic-pituitary-adrenal axis. (B) Pathway (Livak and Schmittgen 2001).



While the avian and mammalian stress pathway is largely conserved, individual response to chronic stress in terms of corticosterone response as well as susceptibility to stress and disease varies significantly based on genetic differences and environmental factors (McEwen and Stellar 1993). Single nucleotide polymorphisms (SNPs) are a major source of genetic difference between individuals in a population based on an unexpected nucleotide replacement. A single SNP can have major accompanying physiological changes or none at all. One of the most famous SNP-associated diseases is multiple sclerosis where two mutations within chromosomes 12 and 20 can significantly increase the chance of developing this particular autoimmune disease (Bahlo *et al.* 2009). Not only multiple sclerosis but SNPs are responsible for other stress-related disorders as well. SNPs within stress-related genes have been directly implicated in the dysregulation of the HPA axis increasing the likelihood of post-traumatic stress disorder, major depression, and vulnerability to disease in general (Lian *et al.* 2014; van West *et al.* 2006; Derijik 2009). Knowing the importance of SNPs in affecting the stress response, this study sought to investigate the location and identity of SNPs by sequencing the high stress (HS) and low stress (LS) lines of Japanese quail bred by Dr. Dan Satterlee. After nine generations of selective breeding based on stress response, Dr. Satterlee's high stress line of Japanese quail had a corticosterone response 58% greater than that of the control stress line. The low stress line had a corticosterone response 23% less than that of the control stress line (Satterlee 1988). In order to investigate genes involved in the stress response, genomic DNA samples of birds from both lines were submitted to a regional genomics center at Michigan State University in order to obtain whole genome sequences from individual birds using next generation sequencing (NGS) performed at that center. SNPs were found by comparing those sequences from the published genome sequence of the chicken and quail performed by Dr. Kang.

Although ASXL2 was not identified as a stress-related gene at the beginning of this experiment, the working hypothesis throughout this study was that high SNP rates as well as differential expression of messenger RNA after stress exposure would identify genes actively involved in the stress pathway.

III. Materials and Methods

This particular study was focused not only on identifying stress-related mutations but also determining which genes were differentially expressed under acute and chronic stress in comparison to their respective controls. SNP identification as well as Real Time-Polymerase Chain Reaction were both utilized to accomplish these aims as outlined in the methodology below. Microarray analysis was performed previously by Dr. Kang yielding 492 genes within the anterior pituitary that were "differentially expressed" in reaction to ten consecutive days of one hour immobilization stress. Ingenuity pathways Analysis was used in order to understand the functions and networks of each of these genes in relation to each other (Kang *et al.* 2012). ASXL2 was one of these differentially expressed genes found after exposure to chronic stress which led to its candidacy for RT-PCR to determine the veracity of the microarray results. The following sections outline the specific methodology used in order to determine if ASXL2 performs a role in the stress pathway.

A.) SNP Identification

Based on data previously generated by Dr. Kang's identification of genetic marker genes, the actual location of the SNPs within the introns and exons of ASXL2 were found utilizing ASXL2 sequencing data from Pubmed (Caldwell, *et al.,* 2005). SNP locations had been previ-

ously found utilizing DNASTAR's SeqMan Ngen Assembly. The locations generated by DNASTAR included a contiguous position in reference to the SNP in the quail genome and a synonymous reference position, identifying the SNP in the chicken genome. Table 2 highlights the expected nucleotide within ASXL2 as well as the SNP allele currently found in the low stress line. The locations of these mutations as well as the expected nucleotides were verified utilizing ASXL2 sequencing data on Pubmed.

B.) Primer Design for RT-PCR

Utilizing Primer3plus, five primers were designed to be less than 200 base pairs and to cover multiple exons along with targeting the more reliable UTR region. These primers were re-constituted with deionized water after received in lyophilized form from the IDT company. After combining each of the primers with the master mixture consisting of the PCR product, Magnesium Chloride, and other components, gel electrophoresis was performed in order to facilitate primer optimization. From Table 1, primer pair #20 was chosen as the best primer to amplify ASXL2 mRNA product to cDNA since it had the highest intensity on the gel electrophoresis slide. Ethidium Bromide was utilized in order to determine the intensity for each of the primers. An image of the gel electrophoresis slide can be seen as Figure 2 in the results section.

Table 1 – Forward and Reverse Primers chosen for ASXL2 based on PCR product quality. Primers were designed utilizing Primer3Plus as well as the chicken (*Gallus Gallus*) reference sequence on Pubmed. Integrated DNA Technology (IDT) was used to order physical primers in lyophilized form.

Numeric Label	Forward Primer	Reverse Primer	Region	Size (bp)
19	gcgaggaaggatgcagac	ggatgccgtaatctgagaa	Exon 1	147
20	aactggaaggtgctgagtt	ccgcccttacaacaaggtta	5' UTR	165

C.) RT-PCR

For RT-PCR, a mixture of 20 microliters of RNA (100 ng/mL), 4 microliters of primers, four microliters of dNTP, and 12 microliters of water was created and placed into a thermocycler, PTC-100, after mixing. After RNA was denatured, it was put in ice to anneal. A 40 microliter cDNA mixture consisting of 10x RT buffer, 25 mM of $MgCl_2$, 0.1 M DTT, RNase OUT, and Superscript III was added to each sample. This new mixture was placed in the PTC-100 thermocycler at 50 degrees Celsius for 55 minutes. Two primers, both a backwards and forwards primer, were then added to this new mixture. A 7500 RT-PCR system from Applied Biosystems facilitated RT-PCR, and 7500 software v.2.0.6 was used to visualize the data for the four sample sets: brain acute control, brain acute stress, brain chronic control, and brain chronic stress.

D.) $\Delta\Delta C_T$ methodology

$\Delta\Delta C_T$ methodology was utilized to quantify the mRNA expression along with β-Actin as the normalized factor of reverse transcription efficiency (Livak and Schmittgen 2001). The average cyclic threshold (Ct) value for β-actin was subtracted from the acute and chronic stress Ct values to determine ΔC_T. Once the $\Delta\Delta C_T$ value was found which resulted in a value of 0 for the controls and then varying values for acute and chronic stress, these values were normalized utilizing the equation $2^{-\Delta\Delta CT}$. After these values were normalized, β-Actin acted as an internal control set at a value of 1.0 to investigate fold changes in expression levels for ASXL2 under acute and chronic stress conditions. Tables 3 and 4 include the C_T values found as well as the normalized expression of ASXL2 appearing down regulated under acute stress and up regulated under chronic stress. *P*-values were found in order to determine the statistical significance of the normalized values for mRNA expression.

IV. Results

A.) SNP Identification

From previous sequencing done by Dr. Kuenzel's lab, four SNPs were identified in the low stress line of ASXL2 seen in Table 2 below. The reference base refers to what the SNP should be while the SNP allele refers to the mutation that is actually observed. Homozygous variant SNPs involve only one extra allele present at the site of the mutation while multiple alleles are possible at the site of the mutation for heterozygous variant SNPs. SNP rates of 75% or higher were considered to be reliable genetic markers for this study (Kang *et al*. 2014).

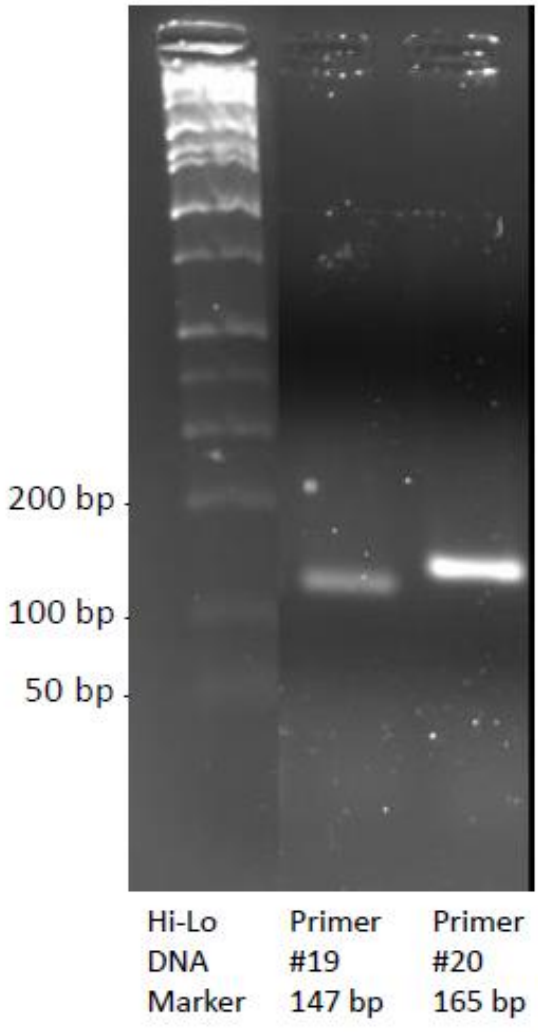
Table 2 – SNP Identification within the Low-stress line of Japanese fowl. The expected nucleotide within ASXL2 as well as the SNP allele currently found in the low stress line of Japanese quail can be seen below. The locations of these SNPs along with the expected nucleotides were verified utilizing ASXL2 sequencing data on Pubmed.

SNP #	SNP location	SNP location (bp)	Reference Base	SNP allele	Genotype	SNP %
SNP #1	Intron #1	104495372	A	G	Homozygous Variant	88.0
SNP #2	Intron #1	104495361	C	T/C	Heterozygous Variant	75.0
SNP #3	Exon #4	104464559	T	C	Homozygous Variant	94.1
SNP #4	Intron #5	104449267	A	G	Homozygous Variant	93.8

B.) Primer Optimization

Figure 2 below depicts the gel electrophoresis slide developed for both sets of the ASXL2 primers.

Figure 2- Expression of mRNA for PCR-Primers in chicken anterior pituitary to determine primer optimization utilizing Gel Electrophoresis; Hi-Lo DNA Marker was used in Lane 1 as a general purpose molecular weight marker for the two primers designed in Lanes 2, and 3. (October 6, 2014)



In the third column, primer pair 20 can be seen exhibiting the highest intensity in comparison to the middle column containing primer pair 19. Although primer #20 is heavier than primer #19 and thereby would naturally have a slightly higher intensity, primer #20 has an intensity exceeding primer #19 by over two-fold. Therefore, primer #20 more efficiently amplified the PCR product. In Lane one, the Hi-Lo DNA Marker was utilized to identify the primers designed by their size or number of base pairs.

C.) Quantification of Gene Expression

In regards to determining the amount of mRNA expressed by ASXL2 utilizing RT-PCR, the null hypothesis was rejected for both acute and chronic stress data with p-values of 0.001345 and 0.00003865 respectively. The data showed that ASXL2 was up regulated under conditions of acute stress and down regulated under conditions of chronic stress. Figure 3 was generated utilizing the $2^{-\Delta\Delta Ct}$ method in order to determine the expression of ASXL2. The ΔC_T value was found by subtracting the control ΔC_T value or the ΔC_T value from the ASXL2 ΔC_T value. The $\Delta\Delta C_T$ value for the acute and chronic stress groups was found by subtracting the average acute or chronic stress control ΔC_T value from the corresponding acute or chronic stress ΔC_T value. The $\Delta\Delta C_T$ value for the acute and chronic control groups was found simply by subtracting the ΔC_T value from itself. The $\Delta\Delta C_T$ value was then normalized as $2^{-\Delta\Delta CT}$ with the control group having a relative quantity value of 1.00. Tables 3 and 4 both highlight the use of $\Delta\Delta C_T$ methodology to quantify the expression of ASXL2.

Table 3- Expression of ASXL2 mRNA in the anterior pituitary under conditions of acute stress (AS), one hour of restraint-induced stress, chronic stress (CS), one hour of restraint-induced stress over 10 days, acute control (AC), and chronic control (CC). Utilizing the $\Delta\Delta C_T$ method, β-Actin acted as an internal control set at a value of 1.0 to investigate fold changes in expression levels for ASXL2 under acute and chronic stress conditions.

Wells	Conditions	ASXL2 C_T	Av. β-actin C_T	ΔC_T ASXL2	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
E1	AC	28.00	20.35	7.66	0	1.00
E2	AC	27.94	20.35	7.59	0.00	1.00
E3	AC	28.00	20.35	7.65	0.00	1.00
E4	AC	27.96	20.35	7.61	0.00	1.00
E5	AC	28.00	20.35	7.66	0.00	1.00

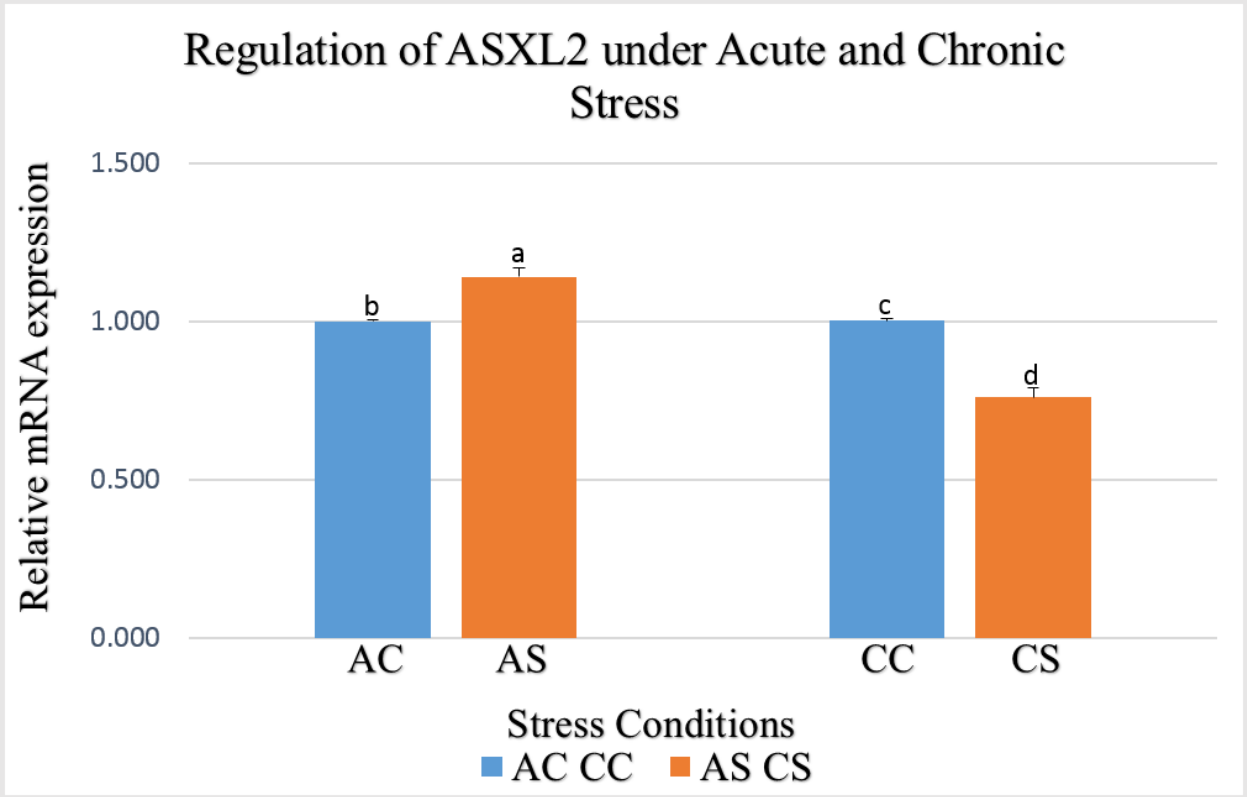
E6	AC	27.99	20.35	7.64	0.00	1.00
F1	AS	27.79	20.31	7.49	-0.15	1.11
F2	AS	27.75	20.31	7.44	-0.19	1.14
F3	AS	27.63	20.31	7.33	-0.31	1.24
F4	AS	27.70	20.31	7.40	-0.24	1.18
F5	AS	27.72	20.31	7.42	-0.22	1.16
F6	AS	27.92	20.31	7.62	-0.02	1.01
G1	CC	27.63	20.25	7.37	0.00	1.00
G2	CC	27.81	20.25	7.56	0.00	1.00
G3	CC	27.71	20.25	7.45	0.00	1.00
G4	CC	27.57	20.25	7.32	0.00	1.00
G5	CC	27.85	20.25	7.60	0.00	1.00
G6	CC	27.73	20.25	7.48	0.00	1.00
H1	CS	28.58	20.78	7.80	0.34	0.79
H2	CS	28.56	20.78	7.78	0.31	0.80
H3	CS	28.61	20.78	7.83	0.36	0.78
H4	CS	28.67	20.78	7.89	0.43	0.74
H5	CS	28.79	20.78	8.01	0.54	0.69
H6	CS	28.73	20.78	7.95	0.49	0.71

Table 4- Expression of ASXL2 mRNA in the anterior pituitary under conditions of acute stress (AS), one hour of restraint-induced stress, chronic stress (CS), one hour of restraint-induced stress over 10 days, acute control (AC), and chronic control (CC). Utilizing the $\Delta\Delta C_T$ method, β-Actin acted as an internal control set at a value of 1.0 to investigate fold changes in expression levels for ASXL2 under acute and chronic stress conditions. A p-value of <0.05 was used to determine the significance of the data.

Stress type	Mean Fold Change in Gene Expression	SE	P-value between groups
AC	1.00	0.007	
AS	1.14	0.031	0.0013
CC	1.00	0.030	
CS	0.76	0.019	0.00004

After normalizing the expression values with $\Delta\Delta C_T$ analysis, Figure 3 was generated in order to visualize the up regulation of ASXL2 under acute stress, and the down regulation of ASXL2 under chronic stress.

Figure 3 – Expression of ASXL2 mRNA in the anterior pituitary under conditions of acute stress (AS), one hour of restraint-induced stress; chronic stress (CS), one hour of restraint-induced stress over 10 days; acute control (AC); and chronic control (CC). Utilizing the $\Delta\Delta C_T$ method, β-Actin acted as an internal control set at a value of 1.0 to investigate fold changes in expression levels for ASXL2 under acute and chronic stress conditions. A p-value of <0.05 was used to determine the significance of the data.



V. Discussion

In terms of importance to this study, ASXL2 has been found to play a key role in epigenetics through its gene products and interactions. ASXL2 encodes a protein that enhances the activity of the trithorax and Polycomb group genes which in turn controls chromatin activation and silencing through histone modifications (Baskind *et al.* 2009). Polycomb and Trithorax gene products are part of distinct multiprotein complexes implicated in regulating transcription by

changing the shape of chromatin to either its active open form or its repressed closed form (Mahmoudi *et al*. 2001). The acetylation of amino-terminal histones opens up the chromatin by "loosening up the interactions between neighboring nucleosomes" while under-acetylated histones are often associated with silenced heterochromatin (Mahmoudi *et al.* 2001). ASXL2 has been found to interact with the deubuiquitinase-coding gene BAP1 which also functions as an epigenetic modifier (Dey 2012). Acute and chronic stress can lead to epigenetic changes in organisms which warrants further study of ASXL2 in light of its up regulation under acute stress and down regulation under chronic stress conditions.

In regards to why ASXL2 mRNA expression was affected by acute and chronic stress, the many functions of ASXL2 in promoting osteoclastogenesis, enhancing PPARγ receptor activity, and regulating adipogenesis all serve as possible explanations for ASXL2's role in the stress response.

Calcium regulation is one of the many ways in which the anterior pituitary mediates the effects of chronic stress. In general, ASXL2 is a key regulator of osteoclastogenesis and bone mineral density, both of which are affected by conditions of hypercortisolism (Farber 2011; Reini 2010). In the brain, the gene product trithorax is activated under conditions of hyper calcium levels seen in neuronal necrosis (Liu 2014). The activation of chromatin-modifying products that are controlled by ASXL2 implicates ASXL2 in the stress response since hypercortisolism can significantly increase the concentration of calcium in the anterior pituitary. A similar activation of trithorax affecting the regulation of ASXL2 could occur in the anterior pituitary as seen in the brain meriting further study.

ASXL2 expression enhances the activity of peroxisome proliferation-activated gamma receptors (PPARγ) (Park *et al.* 2014). Elevated PPARγ levels have been found to reduce HPA

axis hyperactivity in multiple parts of the brain including the brain cortex and the hippocampus (Ulrich-Lai *et al.* 2013). The down regulation of ASXL2 during chronic stress may be attributable to habituation. With the down regulation of ASXL2, the subsequent production of peroxisome proliferation-activated gamma receptors is also decreased thereby limiting the body's ability to modify the effects of hypercortisolism (Ulrich-Lai *et al.* 2013).

ASXL2 promotes adipogenesis and is a key regulator in the formation of white adipocytes (Cristancho and Lazar 2011). Multiple studies have found a key relationship between HPA activation and the expansion of visceral adipose tissue (Mohammed-Ali 1998). Furthermore, adipose tissues have been known to metabolize glucocorticoids (Mohamed-Ali 1998). Therefore, the activation of ASXL2 is potentially part of the body's response in processing increasing amounts of cortisol under stressful conditions.

According to the 2011 survey "Stressed in America" conducted by the American Psychological Association, 44 percent of Americans surveyed believed that "their stress has increased in the past 5 years" with almost a third of children surveyed reporting physical symptoms in response to stress in their lives (APA 2011). With such growing numbers taking a toll on both the physical and mental health of everyday Americans, it is more important than ever to understand the genetic basis for chronic stress and to be able to identify those at risk. ASXL2 is one from among many genes that demands further research in order to further understand the stress pathway as well as to combat an epidemic of chronic stress in our society.

In conclusion, the presence of stress-associated SNPs within ASXL2 as well as its differential expression under chronic and acute stress conditions support our hypothesis that it is involved in the avian stress system and thereby potentially associated with the human stress system.

VI. Acknowledgements

I would like to thank all of the members of Dr. Kuenzel's lab, particularly Dr. Kang for all of his assistance the last three semesters. I would also like to thank the National Science Foundation for supporting my project through an REU supplement. The National Animal Genome Research Support Program (NRSP8) provided the funding to obtain Next Generation Sequencing of the two lines of Japanese quail.

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