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Integrated study on the mRNA expression of the human serotonin transporter

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INTEGRATED STUDY ON THE MRNA EXPRESSION OF THE HUMAN SEROTONIN TRANSPORTER

by

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CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

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has been approved by the Examining Committee
for the thesis requirement for the Master of
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To my mom, Sita SP Subramaniam; my uncle, Vijayanandan Rajendram; the love of my life, Tim Kent Dogan; and my lovely sister, Diveena Vijayendran;
you have always believed in me and I am forever grateful.

An investment in knowledge pays the best interest.

Benjamin Franklin

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CHAPTER 1. INTRODUCTION

Serotonin Neurotransmitter

Serotonin, also known as 5-hydroxytryptamine (5-HT) is a member of the monoamine family of neurotransmitters. Serotonin has been demonstrated to have a complex regulation role in numerous behavioral and neuropsychological processes including impulsivity [1], aggression [1] , appetite [2], memory [3], and mood [4]. In addition, serotonin plays a critical role in integrating signaling pathways involving other non-monoaminergic transmitter systems. Serotonin has a prominent role in health and human development. It has a conspicuous role in regulating early neural (brain) development and adult neuroplasticity including, but not limited to cell proliferation, migration, and programmed cell death [5]. Dysfunction in serotonergic signaling has been associated with mental disorders such as schizophrenia, obsessive-compulsive disorder, and panic disorder [6]. The synthesis and catabolism of serotonin is a highly conserved process. In human, this biogenic monoamine is derived from an aromatic amino acid precursor, L-tryptophan, in peripheral cells such as those in the intestinal ganglia or megakaryocytes, as well as in serotonergic neurons. Because humans cannot synthesize L-tryptophan, supply of this amino acid precursor is a critical regulatory checkpoint.

However, other steps are also important in serotonin synthesis and catabolism as depicted in Figure 1. After hydroxylation of tryptophan, 5-hydroxy-L-trypthophan is decarboxylated by *aromatic amino acid decarboxylase* to finally form 5HT. However, once created, 5HT can also be metabolized by the mitochondrial enzyme *monoamine oxidase A* (MAOA), yielding a serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA) [7]. *Monoamine oxidase A* (MAOA) is assumed to primarily metabolize serotonin although *monoamine oxidase B* (MAOB), which has a lower affinity for 5HT, is also present in serotonergic neurons [8]. Through manipulation of the interplay between

synthesis and catabolism, cells and organisms exert control of basal levels of 5HT. Like most neurotransmitters, the cellular location of 5HT is compartmentalized. After synthesis, cytosolic serotonin is taken up into synaptic vesicles by a vesicular monoamine transporter and packaged into vesicles until exocytosis. Following an action potential, serotonin can be released to the synapse and therefore interact with both the pre and post-synaptic receptors. After release, the synaptic action of serotonin can be terminated via the reuptake action performed by the serotonin transporter or by diffusion out of the synapse [6]. The role of the serotonin transporter in serotonergic neurotransmission is illustrated in Figure 2.

The Serotonin Transporter

The serotonin reuptake transporter (SERT) consists of 630 amino acids [9], and is an integral membrane protein with twelve membrane spanning domains. The transporter protein reuptakes serotonin from the synapse and carries it across the plasma membrane into the pre-synaptic neuron, where it can be re-cloistered in vesicles. Human neuronal, platelet, placental, and pulmonary membranes contain serotonin transporters [9]. In the brain, serotonin transporters are primarily concentrated in the midbrain, basal ganglia, and thalamus, with lower levels of expression in the limbic system, and in the cortex [10-12]. This transporter is highly evolutionarily conserved, modulating serotonin concentrations in the extracellular fluid and hence, regulating the serotonin receptors and the serotonergic neurotransmission system [13]. Hence, the SERT is a prominent candidate locus for studying and understanding gene-environmental interactions in psychiatric neuroscience due to the ability of serotonin to orchestrate neurotransmitter systems.

Interstitial serotonin has the ability to interact with the pre- and post-synaptic neurons. Since the transporter functions as a reuptake transporter, it regulates the extent and duration of spatial interaction between the serotonin neurotransmitter and the neurons. Prolonged interaction time prevents further signal from being transmitted while

active removal of serotonin decreases diffusion and constrains interaction area. Therefore, it is of no surprise that selective serotonin reuptake inhibitors (SSRIs) antidepressants such as fluoxetine (Prozac) and sertraline (Zoloft) that are prescribed to patients with neuropsychiatric disorders target the reuptake transporter.

Like many transport processes, 5HT reuptake is a sodium dependent process with both extracellular sodium (Na+) and chloride (Cl-) ions facilitating the reuptake process. The serotonin transporter has a high affinity for these ions. Like all processes that depend on Na+/K+ ATPase activity, intracellular potassium ions (K+) also play a critical role in this process. An established concentration gradient of these ions is the driving force for the energetically unfavorable [14] transport of serotonin in the presence of the sodium-potassium adenosine triphosphates (Na+/K+ ATPase) enzyme [15, 16]. Energy for the thermodynamically unfavorable transport of the serotonin substrate from a lower concentration at the synapse to a higher concentration in the pre-synaptic neuron is feasible due to the Na+ transport along the electrochemical gradient [14]. Detailed illustration of the co-transport is shown in Figure 3.

The Serotonin Transporter Gene and the Serotonin Transporter Linked Polymorphic Region (5HTTLPR)

In humans, the *serotonin transporter* gene (SLC6A4) encodes SERT. This gene is situated on the human chromosome 17q11.2 [9] and consists of 14 exons that span approximately 42, 000 base pairs. However, this exon count is slightly misleading as the area referred to as exon 1 is actually composed of three distinct DNA segments referred to as exons 1A, 1B, and 1C. These non-coding exonic segments are alternatively spliced. This alternative splicing occurs at the 5'UTR. Initially, these splice variants were discovered using samples from the rat brain stem where 5' rapid amplification of cDNA ends (5'RACE) were performed to determine variations in SLC6A4 mRNA expression [17]. As a result, two major splice variant products (1A+2 and 1A+1B+2) were detected

in a tissue-specific manner utilizing real time polymerase chain reaction (RT-PCR) [17]. The shorter mRNA is exon 1A+2. This shorter product was expressed higher than exon 1A+1B+2 in the adult central nervous system (CNS) and the adrenal medulla, while being equally expressed in the stomach and heart [17].

Although a third splice variant, 1C+2, was not detected during these earlier 5'RACE procedures, this variant was identified subsequently using RT-PCR. Since 1C+2 was only expressed in the gut and in very small amounts in the heart, it was speculated that this splice variant may very well be utilizing a different promoter than the other two splice variants [17, 18]. This rat model can be used as a basis for studying and understanding the differential splicing of exon 1 because the nucleotide sequence of exon 1 is highly conserved between mice and humans [17, 19]. Nucleotide conservation analysis was also used to further validate the presence of exon 1C in humans. A region upstream of exon 1B in the human SLC6A4 gene has a 68% identity to the sequence of exon 1C in rat, implying that the presence of the third splice variant in human is absolutely probable [18]. Thus far, human SLC6A4 gene has only been shown to encode for splice variants 1A+2 and 1A+1B+2 [18, 20]. An mRNA species consisting of only exon 1B+2 was originally detected [20] but has not been reported subsequently to date.

In humans, a GC-rich functional polymorphic region, known as the serotonin transporter linked polymorphic region (5HTTLPR) is present in the 5' promoter region, approximately 1, 400 base pairs upstream of the transcriptional start site (TSS) of the SLC6A4 gene [21-23]. This extensively studied domain acts as the transcriptional control region of the gene by modulating transcriptional activity.

Numerous studies have examined the role of the 5HTTLPR in the regulation of SLC6A4 function. As the name implies, in humans, this region is polymorphic, with the two most commonly studied 5HTTLPR alleles being the short (S) and long (L) alleles. Although there can be other slight differences, the key difference between the S and L alleles is a 43 base-pair insertion/deletion (although initially it was thought to be a 44

base-pair insertion/deletion) [24-29]. The S variant consists of 14 repeat elements whereas the L variant has 16 repeat elements [21, 22]. Three genotypes can result from these two alleles: homozygous LL, heterozygous SL, and homozygous SS. The frequency of these genotypes has been shown to vary by population. Genotypes of North American/European Caucasian is 32% LL, 49% LS, and 19% SS [18, 30, 31]. In contrast, about 57% of Asians have the SS genotype [18, 30, 31]. Scientific evidence from multiple studies has continuously established that the polymorphism influences transcriptional activity of the SLC6A4 gene [21, 22], and has an arguably essential role in susceptibility towards multiple disorders. The rate of mRNA transcription in the presence of the S allele is much lower (lower expression of serotonin transporter) than that of the L allele (higher expression of serotonin transporter) in lymphoblast cell lines [23], human placental choriocarcinoma cell line [22], and immortalized raphe cells [32]. In addition, a higher level of mRNA was detected in lymphoblast cell lines with the homozygous LL genotype as opposed to cell lines with at least one copy of the S allele [23]. However, the finding on the difference in transcriptional levels is not unanimous [33].

Besides the S and L alleles, another tandem repeat sequence variant discovered by Delbrück and colleagues [34] is represented as an extra-long (XL) variant and its transcriptional characteristics have yet to be completely deduced. The sequence of the XL variant is complex and consists of deleted S variant segments and duplicated repeat elements, resulting in a significantly larger (~85kb) variant than the L allele [34]. Like both the S and L alleles, the XL allele is in marked population disequilibrium and is particularly enriched in those individuals of African ancestry origin [34]. In contrast to the S and L alleles, the effects of this allele on SLC6A4 gene transcription are unknown. Therefore, given the key role of SLC6A4 in regulating serotonergic neurotransmission, the difficulties of discerning exact effects of the 5HTTLPR in human behaviors delineated in previous studies using only two contrasts, and the differences of this allele from other previously characterized alleles, it may be possible to more exactly determine

the role of the entire locus through careful interrogation of the XL allele to determine the effect of this variant on gene expression.

At the same time, even though the present focus is on the 5HTTLPR, it is important to appreciate the role of genetic variation in other portions of the gene. In addition to the 5HTTLPR, several other aspects of the SLC6A4 gene that has been investigated include the rs25531 (A to G variation) single-nucleotide polymorphism (SNP) at the 5HTTLPR [27, 28], and the variable number tandem repeats (VNTR) polymorphism at the second intron [35]. Figure 4 shows a schematic diagram of the human SLC6A4 gene and several other well-known polymorphisms present in this gene.

Serotonin and Major Depression

As previously mentioned, altered serotonergic function has been linked to numerous neuropsychiatric conditions such as major depression (MD). MD is a very common and recurrent psychiatric disorder that is defined as the sustained presence of anhedonia (loss of interest or pleasure) and/or depressed mood, in the context of other cognitive or neurovegetative symptoms

MD is common. Although the exact numbers vary with respect to methods used, current epidemiological data has shown that 6% of the population will experience a depressive episode in any 12-month period of time while about 16% have a lifetime prevalence of MD [36, 37]. The frequency of MD is gender specific and is twice common in females as compared to males [37, 38]. In addition to its effect on suicide rates and human misery, MD has a significant socioeconomic impact. Murray and colleague [39] have demonstrated that MD is one of the leading contributors to world-wide disability with enormous treatment related costs. In 2000 alone, the total treatment cost in the United States alone was estimated to be $83.1 billion [40].

Despite the large numbers of previous studies, there still is considerable interest among psychiatrists and pharmacologists to better understand the underlying mechanisms

of the SLC6A4 gene and possibly engineer effective medical therapeutic interventions. These efforts are not without controversy. The majority of investigations have supported the hypothesis that variation in serotonin concentrations in the CNS has a relationship to MD. To a large extent, the conclusions of these studies have been buttressed by the overwhelming success of selective serotonin reuptake inhibitors (SSRI's) in the treatment of MD. SSRIs, which include fluoxetine and paroxetine, are a class of antidepressants that target the serotonin transporter, block the reuptake process, and presumably increase the availability of serotonin. However, the fact that altering serotonergic reuptake can alter the occurrence of MD does not necessarily imply that SLC6A4 function is altered in MD. Arguing this point and noting the inconsistencies in some prior studies, a number of investigators have attempted to directly determine the relationship of 5HT or its metabolites in those with depression. Those studies have had mixed results. For instance, Roy and colleagues [41, 42] conducted a longitudinal study showing the significant decrease in 5-HIAA (primary serotonin metabolite) in depressed patients with suicidal tendencies. Additionally, several other studies have reported decreased serotonin concentrations in postmortem brain tissue, and low plasma tryptophan concentrations in depressed individuals. However, Meyer and colleagues [43] did not find any difference in regional serotonin measured using positron emission tomography between depressed and non-depressed individuals. Taken in total, these studies suggest that although valuable, it is likely that any improvement to healthcare as a result of a better understanding of the overall serotonergic function will be incremental.

These observations and conclusions also extend to that of understanding the role of SLC6A4 in serotonergic function. To date, researchers have not been able to reach a consensus through their studies on whether the S allele is associated with depression and most investigators agree that any main effect must be rather modest. Driving this belief is the undisputed fact that the S allele has been shown to have a lower transcriptional efficiency than the L allele.

Increasingly, this belief has given rise to environmentally contextual examinations of the effect of the 5HTTLPR polymorphism on vulnerability to illness. Originally, it was predicted that genetic makeup (underlying DNA sequence variation) was the sole driving force of susceptibility to MD and that heterogeneity in the environment played little role in evoking the effects of the allele on illness. Using this approach in a large number of studies, researchers were unable to accumulate consistent evidence to support a main effect of the 5HTTLPR system on vulnerability to illness. Further exploration led to the hypothesis that the SLC6A4 gene may be moderated by external environmental factors, creating an additional level of complexity. This gene-environment interaction (G × E) idea was certainly more compelling because individuals with the same 5HTTLPR genotype showed similar patterns, but did not exhibit an identical level of susceptibility to MD.

The interaction between genetic and environmental risk factors was examined by Kendler and colleagues [44]. They were particularly interested in understanding the extent of contribution of each risk factor to the onset of major depression. In order to assess this, they proposed two models: additive and genetic control of sensitivity to the environment. The additive model suggests that the genetics and environmental components are independent of one another such that a stressful life event affects individuals with low-risk and high-risk genotypes similarly. However, the genetic control of sensitivity to the environment model implies that the gene modulates the sensitivity of an individual to a stressful event that has the potential to induce depression. In this model, individuals with a high-risk genotype possess a greater risk of developing MD than individuals with a low-risk genotype. The results and analysis of the study supported the latter model, concluding that a positive interaction is present between genotype and environment in the onset of MD.

One of the most influential investigations on the idea that the 5HTTLPR under stressful events could influence susceptibility to MD was conducted by Caspi and

colleagues [45]. They evaluated the G × E interaction in individuals of the Dunedin Multidisciplinary Health and Development Study [45, 46]. Individuals with the LL homozygous genotype were less likely to develop MD than those with the S allele. Experiencing four or more stressful events led about twice the number of individuals with the S allele to depression compared to those with the LL genotype. Similar studies have been carried out in adult twins by Kendler and colleagues [47], in female adolescents by Eley and colleagues [48], in Koreans by Kim and colleagues [49], in longitudinal studies by Wilhelm and colleagues [50], and by Cervilla and colleagues [51], and in a family-based study by Dick and colleagues [52].

The G × E interaction has been also further explored and validated in rhesus monkeys. This non-human primate model can be used as a basis for understanding the interaction between the 5HTTLPR and stressful environment in humans because the 5HTTLPR length variation in rhesus monkeys is analogous to that of humans [53, 54]. In the rhesus monkey study by Champoux and colleagues [53], monkeys were raised in two environments: mother-reared (less stressful) and nursery-reared (more stressful). Monkeys raised in the less stressful environment did not exhibit emotional distress differently regardless of their genotype. However, monkeys with the S allele showed greater distress than monkeys with the LL genotype in the more stressful environment. Bennett and colleagues [55] examined the 5-HIAA concentration in the cerebrospinal fluid of rhesus monkeys in two environments as well. In the stressful environment, 5-HIAA concentrations were lower in monkeys with the S allele compared to those with the LL genotype. No difference in concentrations was noticed in the less stressful environment. These studies complement each other and certainly validate the interaction between the 5HTTLPR and stressful environments resulting in phenotype developments.

Although G × E interactions have expanded our knowledge and helped answer several critical questions, ultimately it is important to delineate the exact mechanism(s) of this interaction in order to fully identify risk factors for prevention, diagnosis, and

treatment of MD. One level of additional intricacy beyond the G × E interactions is epigenetics. Epigenetics is the "modifications in gene expression that are brought about by heritable, but potentially reversible, changes in chromatin structure and/or DNA methylation" [56]. DNA methylation is an essential biochemical process for cellular differentiation and development of an organism. It generally occurs in a CpG dinucleotide where the 5 position of the cytosine pyrimidine ring has a methyl group attached to it. DNA methylation has the ability to stably alter gene expression and they are often inversely correlated [57, 58]. Clusters of CpG dinucleotide known as CpG Islands are typically situated in the promoter region of a gene, hinder transcriptional factors from binding and attract methyl-DNA binding proteins, leading to gene silencing [58, 59]. Two common epigenetic silencing that are interceded by DNA methylation are gene imprinting and X-chromosome inactivation [60].

Several studies have taken the 5HTTLPR genetic variation, external environment, and epigenetic factors into consideration to better address variations in phenotypes. A study by conducted by Philibert and colleagues [61] on human lymphoblast cell lines revealed that females had higher CpG methylation than males. They also observed that lifetime history of MD was correlated to an increase in overall DNA methylation. The study by IJzendoorn and colleagues [62] evaluated individuals whom have experienced loss or other traumatic events. A relationship was deduced between the 5HTTLPR genotype, unresolved state of mind (USM), and methylation density. Higher methylation in the presence of the L variant resulted in more USM. For the S variant, lower methylation resulted in more USM. However, unexpectedly, the SS genotype under higher methylation showed less USM. This demonstrates the interplay between epigenetic factors and genetic variation in determining phenotypic outcomes.

Serotonin and Child Maltreatment

In an attempt to better understand and elucidate developmental mechanisms, researchers have resorted to a more elaborate analysis, taking into account all possible factors that could potentially explain these mechanisms. Considerable importance have been placed on studying child maltreatment, including physical abuse, neglect, and sexual abuse, due to the adverse short-term and long-term consequences of these acts. Multiple studies have demonstrated that child abuse affects physical, social, and cognitive development in numerous levels in abused individuals. As a result of physical abuse, individuals have reported insecure attachments [63-65], lack in social interaction with peers [64-68], and inferior cognitive maturity [65, 68, 69]. Similarly, sexual abuse has led to somatic complaints primarily in boys [65, 70], improper sexual behavior [65, 70, 71], retraction of social interaction [65, 70, 71], and developmental impediment primarily in girls [65, 70].

Child maltreatment not only hinders physical, social and cognitive development, but it also places individuals at a high-risk for being vulnerable to and developing maladaptive behaviors and psychopathology, including alcohol and/or drug abuse [65], anxiety [65, 72-74], antisocial tendencies [65], and depression [65, 74, 75]. Because the exact nature of maltreatment differs between individuals and each individual's response to the abuse is dependent on their prior experience, maltreatment may open up an entirely unique epigenetic path for each affected individual. However, it is generally believed that many of these effects will involve common pathways and that a more exact understanding of the genetic variations, G × E interactions, and epigenetic factors could be beneficial in developing therapeutic interventions.

This has naturally led to the examination of GxE effects with respect to the serotonergic system. 5HT has been hypothesized to be a transmitter more easily affected by environmental perturbations, resulting in alteration of neural information processing. This biased processing could result in the destabilization of behavior, cognition and affect

can be destabilized, which normally are coupled under the regulatory function of serotonin in normal development [76, 77]. Therefore, it can be hypothesized that child maltreatment that causes abnormal behavior could exert its effects through factors that are associated with the regulation of serotonergic neurotransmission. Given the prior evidence that both serotonergic neurotransmission and child maltreatment are linked to depression, this makes the 5HTTLPR of the SLC6A4 gene an excellent locus for studying G × E interactions.

Several studies have investigated the interaction between the 5HTTLPR and child maltreatment. Caspi and colleagues [45] demonstrated that individuals with the S variant exposed to childhood maltreatment have a higher risk of developing adult depression than those with the LL genotype exposed to childhood maltreatment. This and other studies have supported the hypothesis that the G × E interactions of the 5HTTLPR with maltreatment over the course of development affect long-term phenotypic and pathophysiological outcomes. Though negative environments like child maltreatment are known for increased vulnerability to depression, positive environments like social support have the ability to decrease the associated risk. This was evident in the study conducted by Kaufman and colleagues [78] where individuals possessing the SS genotype with a history of maltreatment that had social support, were less likely to develop depression compared to those with the SS genotype with a history of maltreatment that did not have any social support. Hence, even if a genetic predisposition is present, degree of impairment can be moderated by environmental factors.

These effects on vulnerability to MD may be moderated through altered DNA methylation. Beach and colleagues [79] reported that the level of methylation of the CpG island upstream of the SLC6A4 gene was associated with childhood abuse in males and females. Beach and colleagues replicated their prior work [80] and in addition determined that in females, childhood sex abuse was significantly correlated to methylation at the promoter region of the SLC6A4 gene, and symptoms of antisocial personality disorder

was significantly associated to methylation at the SLC6A4 gene. Since gene transcription is modulated by DNA methylation, it is apparent that epigenetic factors play a conspicuous role in regulating genes and consequently the prevalence of diseases.

The focus of this thesis

In spite of extensive study for many years on the SLC6A4 gene, the mechanisms underlying the regulatory control of this gene still remains poorly constrained. Furthermore, since this locus is the target of virtually every antidepressant, the exact molecular mechanisms of action of these therapeutic agents could provide valuable insight on preferential response to these agents on an individual basis. Therefore, it is important to further investigate these mechanisms governing the biological variations in the amount of serotonin transporter protein as this could lead to the development of more effective chemotherapeutic interventions and aid the advancement in personalized health care.

In an effort to accomplish this task, it is absolutely necessary to understand factors contributing to the production of the serotonin transporter protein. The amount of protein produced is intrinsically related to the total mRNA of the SLC6A4 gene. The transcription of this gene is known to be regulated by both genetic and epigenetic factors. In order to facilitate a better understanding of these factors, the gene expression and gene methylation of previously genotyped lymphoblast cell lines were determined. The role of child maltreatment and depression on genotype, gene expression and gene methylation was of particular interest. Moreover, since gene expression is a critical tool in understanding cellular behavior, the normalization of gene expression in the presence of multiple housekeeping genes was explored. Experiments were performed with the generalized hypothesis that the SLC6A4 gene expression is regulated by the interaction between genotype, gene methylation and external environmental factors.

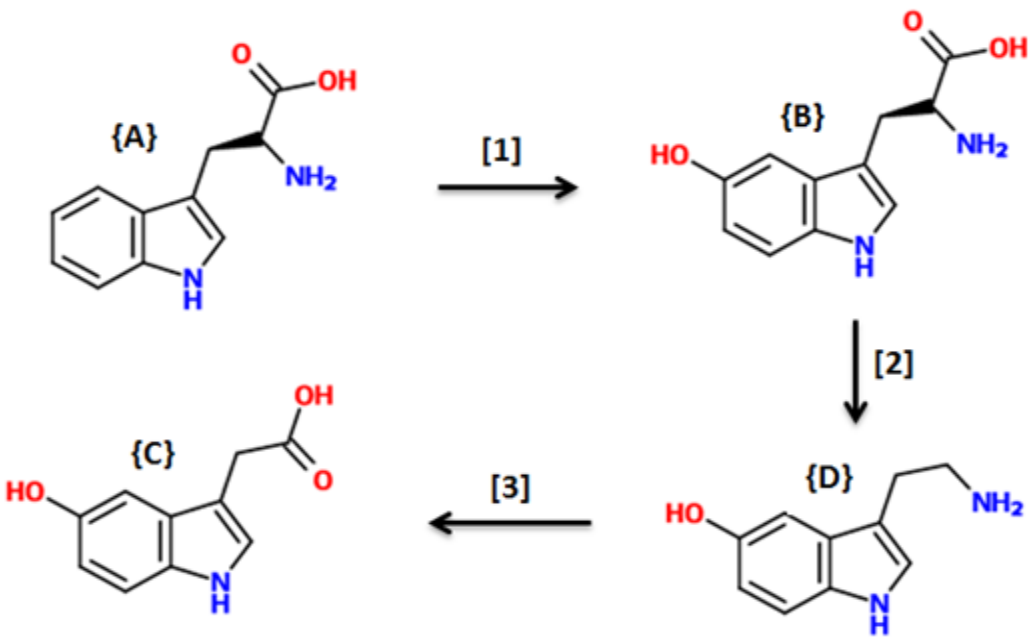

Figure 1. The synthesis of serotonin. Initially, L-tryptophan {A} is converted to 5-hydroxy-L-tryptophan {B} in the presence of tryptophan hydroxylase [1]. 5-hydroxy-L-tryptophan is then converted to serotonin {D} in the presence of an amino acid decarboxylase [2]. Serotonin is further metabolized to 5-hydroxyindoleacetic acid {C} through the action of monoamine oxidase A [3].

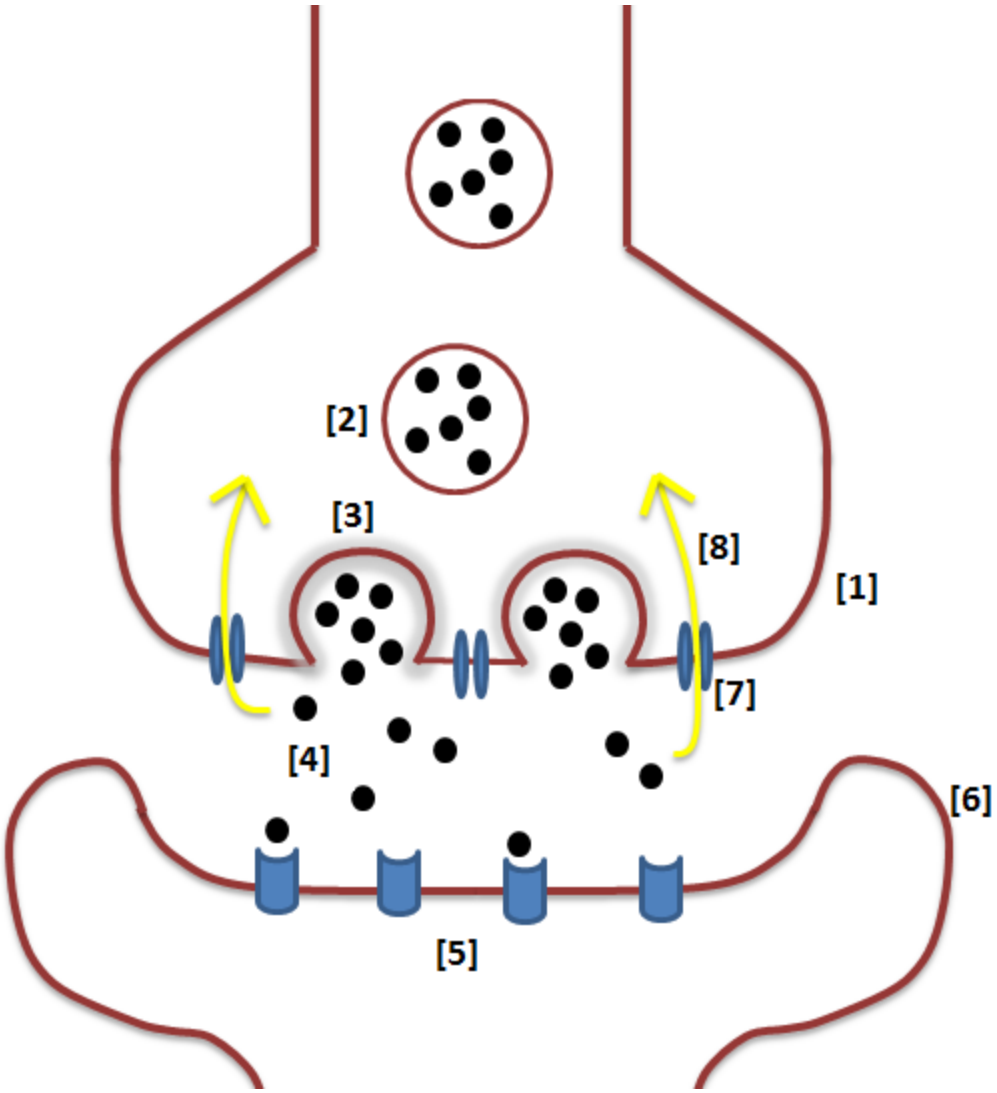

Figure 2. This figure depicts the serotonergic neurotransmission. Serotonin in the pre-synaptic neuron [1] are cloistered in vesicles [2] and transported. The vesicle fuses with the pre-synaptic neuron [3] and is consequently released to the extracellular space (synapse) [4]. Interstitial serotonin binds to the receptors [5] at the post-synaptic neuron [6]. Serotonin reuptake transporters [7] present at the pre-synaptic neuron reuptakes serotonin [8] from the extracellular space back into the pre-synaptic to be re-cloistered in vesicles.

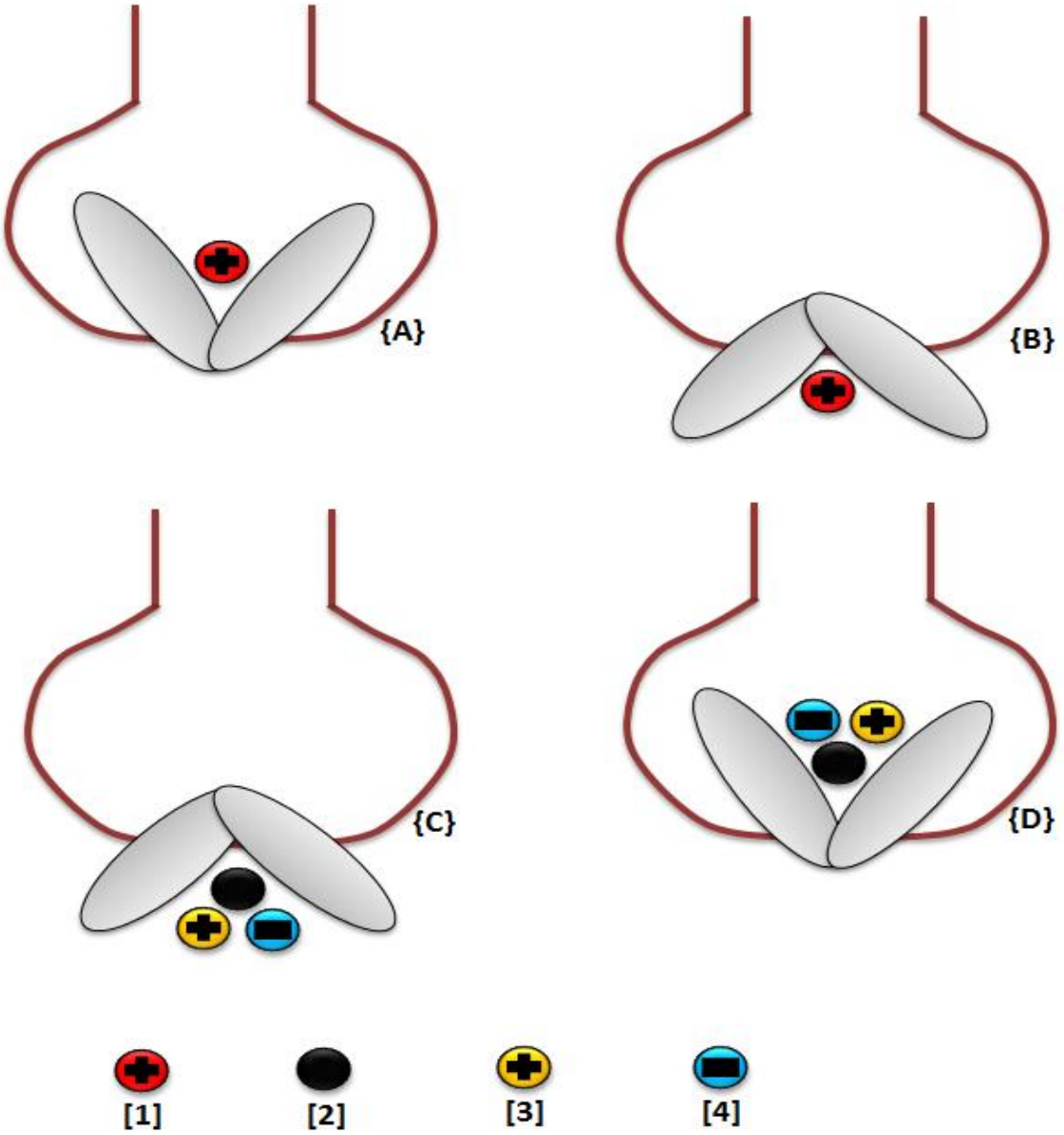

Figure 3. The serotonin reuptake transporter facilitates the reuptake process of serotonin. An intracellular potassium ion [1] binds to the serotonin transporter {A}. Conformational changes due to this binding allow the transporter to release the potassium to the extracellular space {B}. Serotonin [2], a sodium ion [3], and a chloride ion [4] are then able to bind to the transporter {C}. Once these three molecules bind, a conformational change occurs, allowing the reuptake process to occur {D}. The cycle then continues.

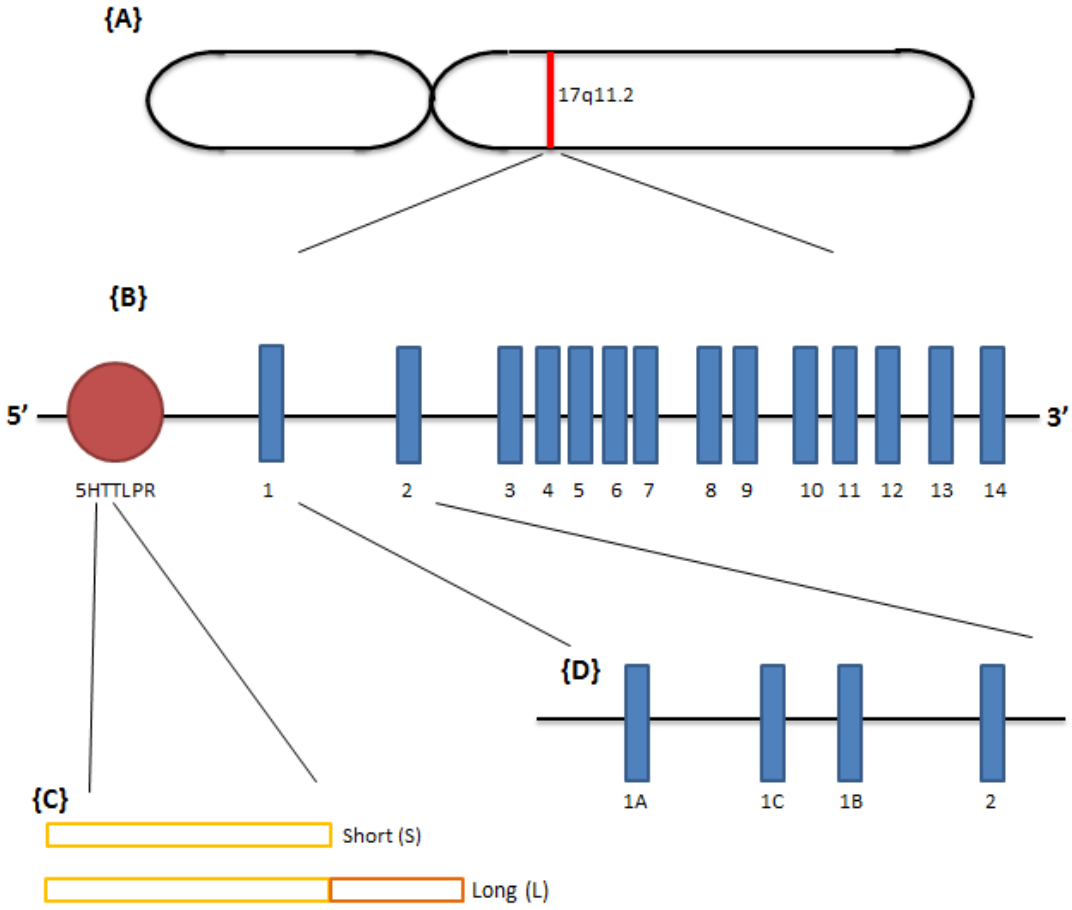

Figure 4. The SLC6A4 gene is located on the human chromosome 17q11.2 {A}. This gene {B} consists of a 5HTTLPR at the 5' promoter region and 14 exons downstream of the promoter. Two common 5HTTLPR variants are the short (S) and long (L) polymorphisms that vary by a 43 base-pair insertion/deletion. Exon 1 of the SLC6A4 gene is variably spliced {D} to splice variants 1A, 1B and 1C.

CHAPTER 2. MATERIALS AND METHODS

Cell culture

Human lymphoblast cells were obtained from the Rutgers University Cell and DNA Repository (RUCDR), (Piscataway, NJ, USA). Cells were maintained in media consisting of Roswell Park Memorial Institute medium (RPMI; Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Gibco-Invitrogen, Carlsbad, CA, USA), Hepes (Gibco-Invitrogen, Carlsbad, CA, USA), and Penicillin Streptomycin (Gibco-Invitrogen, Carlsbad, CA, USA) in a 37ºC/5% CO_2 humidified incubator. Media was changed every other day. The initial flask of cells was eventually split into three T75 flasks. One flask was designated for DNA extraction, another for RNA extraction and the final flask of cells were frozen down in DMSO in nitrogen tanks for future use. The final growth media change was performed approximately 24 hours prior to DNA and RNA extraction. For these studies, a total of 158 Iowa Adoption Study (IAS) [81] and 139 Family and Community Health Study (FACHS) [82] cell lines were used.

DNA extraction, RNA extraction, and genotyping

DNA was extracted using cold protein precipitation with isopropanol [61] while RNA was prepared from the lymphoblast cell lines using the standard protocol of the RNA purification kit by the manufacturer (Invitrogen, Carlsbad, CA, USA). DNA was used for genotyping and methylation profiling whereas RNA was used to obtain the SLC6A4 gene expression of exons 1A+1B and 8. The genotyping of the 5HTTLPR utilizing standard Polymerase Chain Reaction (PCR) and electrophoresis with polyacrylamide gels has been described previously [61, 83].

Gene expression and gene methylation

In order to measure gene expression, total RNA was reverse transcribed to cDNA using the manufacturer's protocol of an Applied Biosystems cDNA archiving kit (ABI, Foster City, CA, USA). This was performed to avoid RNA from degrading rapidly. Quantification of SLC6A4 mRNA expression of exons 1A+1B and 8 was conducted using commercially available primer probe sets and amplification reagents from Applied Biosystems (ABI, Foster City, CA, USA) on a Fluidigm BioMark Genetic Analysis System (South San Francisco, CA, USA) based on the protocol provided by the Fluidigm Corporation. Primer probe sets specific to the SLC6A4 gene sequence bridged exons 1A and 1B (ABI, Hs00984354) and exons 8 and 9 (ABI, Hs00169010) have been previously described [61]. Housekeeping gene probe sets (CALR, UBC, RPL7A, RPS19, RPS 20) were included to account for internal copy number variations. Following pre-amplification as stated on the protocol by Fluidigm Corporation, real-time PCR (RT-PCR) quantification was performed in triplicates in a 96 by 96 dynamic integrated fluidic circuit array on the BioMark Fluidigm platform at default settings. Two independent runs were conducted and resulting C_T counts were determined using proprietary Fluidigm software. Extracted results were exported to Microsoft Excel (Microsoft Corporation, USA) for data management. Gene expression data were converted into Z-scores by subtracting the mean of all samples from the C_T count of an individual sample and consequently dividing the new value with the standard deviation of the data set. This allows for comparison of gene expression values between samples.

Due to the inability to construct a Taqman probe for splice variant 1A+2, Sybr Green based RT-PCR was performed to assess the presence of this variant. Custom primers for this assessment included the forward primer CCGAGCTCTCTATCGTCGGGATTGACACGTCGGGATTGA situated on exon 2 and the reverse primer CCAGCCCGGGACCAGCCTCCCCGCGCAGCC situated on exon 1A. RT-PCR was performed with the aforementioned primers, POWER SYBR Green

Master Mix (ABI, Foster City, CA, USA) at an annealing temperature of 70°C on a 7900HT Fast Real-Time PCR System (ABI, Foster City, CA, USA). Finally, the 1C+2 variant was evaluated using the forward exon 2 primer mentioned above and a reverse primer AGCCTCCACGGCGGTGAAATGAAG situated at exon 1C. A positive gene expression profile for this variant was not obtained.

Gene methylation profiling of samples were conducted by the University of Minnesota Genome Center, using the Illumina HumanMethylation450 BeadChip. In this assay, genomic DNA is bisulfite treated in order to detect cytosine methylation at CpG Islands. When the DNA is bisulfite-treated, methylated cytosines are not converted and unmethylated cytosines are converted into uracil. At each locus, two probes are designed. The "M" probe is designed to align with the methylated region whereas the "U" probe is designed to align with the unmethylated region. Methylation level of each sample is determined from the probe intensities where a beta value is the ratio of the methylated probe intensity to the total probe intensity at the locus. GenomeStudio V2009.2; Methylation module Version 1.5.5, version 3.2 (Illumina, San Diego, CA, USA) was used to determine the average beta value at each CpG residue. Average beta values of 16 CpG residues of the SLC6A4 gene was exported to Microsoft Excel (Microsoft Corporation, USA) from a genome wide data set and converted into Z-scores.

Data management of genotype, gene expression, gene methylation and clinical data were done on Microsoft Excel (Microsoft Corporation, USA). Consequently, these data were imported and analyzed on JMP version 9 (SAS Institute, Cary, SC, USA) and SPSS version 19 (IBM, New York, USA) statistical software. The relationship between genotype and gene expression was determined using an ANOVA test on JMP. SPSS was utilized to obtain the interaction effects on DNA methylation. A multivariate model was used.

CHAPTER 3. RESULTS AND DISCUSSION

Relationship of genotype and child abuse on gene expression and gene methylation of the human serotonin transporter

In order to determine the interaction effects of genotype and child abuse on the gene expression and gene methylation of the human serotonin transporter, we extended our analyses of the effects on DNA methylation to include more gene regions and the effects of methylation on differential splice variant gene expression. Experiments were performed with the hypothesis that childhood sexual abuse would result in a genotype dependent alteration of DNA methylation at the SLC6A4 locus.

The clinical, demographic and genetic characteristics of the 158 subjects included in this study are described in Table 1. However, only a subset of 152 subjects was used in the DNA methylation analysis due to missing data points. In summary, the subjects are all female and nearly all white. At the time of the blood draw for the preparation of the cell lines used in these epigenetic and gene expression and the subjects tended to be in their middle to late 40s. At the Wave 4 interview, 16 of the subjects reported sexual abuse by a family member with an additional 10 subjects denying sexual abuse by a family member but reporting sexual abuse by a non-family member. The structure of the SLC6A4 gene and the placement of the 16 methylation probes are illustrated in Figure 5. SLC6A4 is a 25 kb, 14 exon gene located on chromosome 17. Exon one is subdivided into three segments, 1A, 1B and 1C, that are differentially spliced to provide three known mRNA products that include exons 2-14 in addition to their unique portions of exon one (exon 1A+B, 1A and 1C).

First, we examined the levels of total and splice variant specific SLC6A4 expression using RT-PCR and primer probes sets designed to recognize the each of the three splice variants described by Ozsarac and colleagues [17]. Despite several attempts,

we were unable to detect expression of the two variants containing exon 1C or just exon 1B. However, using a previously developed probe set which recognizes the exon 1A+B variant (the MGB probe bridges the junction between 1A and B) and a probe that recognizes all three variants (the MGB probe bridges exon 7 and 6). The relationship of the expression levels of each of these transcripts to 5HTTLPR genotype is given in Table 2. As the table indicates, although the directionality is consistent with prior findings, in this small group of samples 5HTTLPR genotype was only significantly associated with total SLC6A4 mRNA levels (exon 8), but not with the levels of the exon 1A+B containing transcript. In addition, consistent with prior assessments, the level of the exon 1A+B containing transcript was only partially correlated with total mRNA levels (r=0.70).

The relationship between methylation at each of the CpG residues and gene expression is delineated in Table 3. As the table indicates, the expression of the exon 1A+B containing transcript was significantly associated with two consecutive CpG residues in the intronic region immediately adjacent to Exon 1C. In contrast, total mRNA expression was only significantly associated with a methylation at cg25725890, a probe found 375 bp upstream of the transcription start site (TSS). There were no significant relationships between methylation status at any of the probes and 5HTTLPR genotype including at two probes (cg12074493 and cg06841846) which map to the 5HTTLPR repetitive region.

The methylation of the core portion of the CpG Island has determined previously using MALDI-TOF mass spectroscopy. Therefore, in an attempt to understand the reliability of these two types of methylation measures, we compared the current values obtained using the Illumina arrays with previous values. Overall, 4 CpG residues (cg03363743, cg14692377, cg05016953, cg25725890) at this locus were surveyed by both approaches. At each CpG residue, the degree of methylation determined by each method was significantly correlated with the average adjusted r^2 equaling ~0.34.

Two distinct classes of sexual abuse were examined. Results on effects of abuse by a family member and genotype on methylation is delineated in Table 4.

In summary, we conducted iterative examinations of the relationships of gene methylation on SLC6A4 mRNA expression and of genotype and child abuse interaction effects with methylation. Limitations of this work include the small number of subjects, the use of non-CNS biomaterials and the self-report nature of the data.

The overarching goal of this project was to continue the biological conversation as the possible biological mechanisms through which childhood sex abuse leads to adult psychopathology. Whereas it is doubtless that a portion of this increased proclivity for depression and other forms of behavioral illness is engendered at the neuroanatomical level, one of the leading hypotheses is that childhood trauma also leaves an indelible mark on gene transcription. If so, the mechanisms underlying these changes should be demonstrable, and perhaps, remediable. In prior examinations, we demonstrated that the genotypically contextual changes in promoter methylation were associated with self-reports of child abuse but did not analyze the relationship of those changes methylation to SLC6A4 transcription. The connection of changes in methylation to possible changes in gene transcription are critical to the hypotheses specifying molecular changes as a result of child abuse because of the tight connection between levels of mRNA and protein [84]. But despite the relative ease of conducting examinations of peripheral SLC6A4 gene expression, in our published [61] and unpublished examinations, we have not been able to demonstrate a significant relationship between SLC6A4 gene expression and depression and there is a relative paucity of publications in this area and we were led to conclude that on the basis of the extant literature, that there may not be a tight connection between the changes in gene methylation observed in these and other studies and peripheral SLC6A4 gene expression.

In the current study, we again demonstrate significant G x E effects. A possible explanation of this observation is that while abuse may alter the patterns of peripheral

blood methylation, these changes may only affect transcription of CNS specific transcripts. In prior decades, this possibility would be deemed unlikely but over the past several years it has become increasingly clear that although individual variance in methylation may be conserved across tissues [85], secondary to the effects of tissue specific transcription factors and multiple spliced genes, these harmonized changes in methylation may not have synonymous effects on gene transcription [86]. Since SLC6A4 has three known splice variants [17] and we have repeatedly demonstrated that the expression of the exon 1A+B variant is only partially correlated with total mRNA as measured by the probe set that spans the juncture of exon 7 and 8 [61, 87], if the changes observed in DNA methylation in association with child abuse are real, they may be exerting their effects through changes in other, more CNS specific SLC6A4 splice variants. Unfortunately, to the best of our knowledge, the transcriptional repertoire of CNS serotonergic neurons has not been described thus making it difficult to make more exact comparisons of lymphoblast and CNS specific SLC6A4 transcription.

Of significant relevance to the hypothesis that child abuse exerts a part of its effects thorough altered SLC6A4 methylation is that the CpG residue immediately downstream of the 5HTTLPR is the most significantly associated residue in our analyses. Given the wealth of prior analyses that demonstrate the effects of this motif at the transcriptional and interactive levels, this seems only logical. However, the failure of the two immediately upstream (more 5') residues is difficult to understand at first glance. However, it is important to note that these two residues (cg12074493 and cg06841846) map to the repetitive element themselves and the molecular mechanism through which increased VNTR number confers increased transcription is unknown. Therefore, it is quite possible that these probes do not accurately interrogate the methylation status at CpG residues these degenerate sequences or that they do not interrogate the most relevant portion of the VNTR associated haplotype.

Overall, 26 of the 158 (16%) female subjects who participated in the study reported childhood sexual abuse which is somewhat lower than the 22% rate reported in the general population [88]. Somewhat surprisingly, the expansion of the affected status in the sexual abuse analyses to include those 10 individuals who were abused by non-family members but not by family members did not increase the significance of the results. Reassuringly, however, in this second expanded analysis the results continued to identify differential methylation at cg18584905 continued to be significant in all aspects of analysis. Although there are a number of ways to interpret these results, one possible conclusion from these results is that consistent with previous findings [89], the impact of sexual abuse by nonfamily members in this population is not as severe as the impact of sexual abuse by members of one's own family.

This is one of few studies to directly compare the results obtained from the Sequenom mass spectroscopy and Illumina single base extension technologies using aliquots of the same samples of DNA. The results, which show modest concordance between the results at the four residues, suggest that the current results are reliable but suggest that there is room for improvement in at least one of the approaches.

In our original analyses, we only examined the effects of child abuse by family members. In this study, we expand our analyses to include sexual abuse by non-family members. Despite the increased number of affected individuals, the strength of the findings at cg18584905 did not increase. Although many explanations for this are possible, consistent with the findings of Sen and colleagues who found that the severity of stressors had significant effects on the strength of the G x E effects [90], it may well be that sexual abuse by family members has much more severe effects on young women than abuse by non-family members.

In summary, the examination of the effects of childhood sexual abuse on methylation at 16 residues spanning the SLC6A4 gene and, among functional loci, demonstrate a significant genotype dependent effect at cg22584138 and a significant

effect of sex abuse at the adjacent loci cg05951817, residues that are downstream of exon 1A.

Relationship of genotype and depression on gene expression and gene methylation of the human serotonin transporter

In order to determine the interaction effects of genotype and depression on the gene expression and gene methylation of the human serotonin transporter, we performed a depression associated DNA methylation analysis of the SLC6A4 gene on differential splice variant gene expression. Experiments were performed with the hypothesis that depression would result in a genotype dependent alteration of DNA methylation at the SLC6A4 locus.

The clinical, demographic and genetic characteristics of the 139 IAS subjects included in this study are described in Table 5. Subjects were all females, nearly all white and tended to be in their middle to late 40s at the time of blood draw. Maximum count of major depressive symptoms (MD_Max) derived from the self-reported Semi-Structured Assessment for the Genetics of Alcoholism, versions 1 and 2 (SSAGA-I and SSAGA II) was used to group individuals.

The analysis performed was identical to that in the context of childhood sexual abuse discussed earlier in this chapter. Identical primer probe sets were utilized to detect the total and splice variant gene expression. The relationship between the expression levels of each of these transcripts to the 5HTTLPR genotype is given in Table 6. As indicated in the table, only the directionality of exon 1A+1B is consistent with prior findings. Larger Z-scores represent larger C_T counts and hence lower levels of mRNA transcript.

The relationship between methylation at each of the CpG residues and gene expression is delineated in Table 7. As indicated in this table, the significant association

between expression of transcripts and CpG residues was identical to the previous analysis with an additional significant associated demonstrated between the total mRNA expression and methylation at cg05016953, a probe found approximately 112 bp upstream of the TSS. The main effects of genotype and depression alongside the interaction effects on methylation are depicted in Table 8. From this table, the main effect of genotype is only seen at cg10901968 (downstream of the 5HTTLPR) whereas both the main effect of depression and the interaction effect are shown to influence the methylation at cg25725890 (intron downstream of exon 1A) and cg20592995 (distal to 3'UTR).

In prior examinations, we have demonstrated that methylation levels at the promoter region of the SLC6A4 gene are associated with a lifetime history of major depressive disorder. In this work, the analysis was expanded by investigating the interaction effects as it has been established that gene expression undergoes complex regulation by genetic, environmental and epigenetic factors. Although it is evident that main effects still have a conspicuous role, the examination of interaction effects allow researchers to approach the complex disorder of depression with an additional level of intricacy. Nevertheless, it is extremely challenging to model the transcription and methylation patterns in the CNS merely using peripheral blood.

In this study, we again demonstrate significant G x E effects. The methylation residue cg05016953 located downstream of the 5HTTLPR and upstream of the TSS is of particular interest as it is not only significantly associated to the main effects of depression and the interaction effects of depression and genotype, it is also significantly associated to the total mRNA expression of the SLC6A4 gene. Yet, this pattern was not apparent at any other methylation residue suggesting that any effects may be conferred through methylation at and near this residue, but perhaps not through other residues. In addition, depression is continuous and the reliability of self-report symptoms makes quantifying the actual level of depression difficult. Hence, these findings though

promising will need further examination in other samples before they can be generally accepted.

In summary, the examination of the effects of depression on methylation at 16 residues spanning the SLC6A4 gene demonstrate a significant genotype dependent effect at cg10901968, a significant depression and genotype × depression effect dependent effect at cg25725890 and cg20592995.

Relationship between the extra-long variant (XL) of the human serotonin transporter to gene expression in an African American population

In an attempt to better understand the SLC6A4 gene with respect to the African American population, another 5HTTLPR variant known as the extra-long (XL) variant was examined. Since prior studies have suggested several race-by-genotype interaction effects [91, 92], and the 5HTTLPR has been associated with differential gene expression, it can be deduced that there is a possibility for varying serotonin reuptake among different populations. Experiments were performed with the hypothesis that the XL variant would be associated with an increased transcriptional efficiency of the SLC6A4 gene.

The transcriptional efficiency of the XL allele was examined in 134 female individuals from the FACHS study. Material derived from these individuals included 86 cell lines that were homozygous for the long allele, 26 were homozygous for the short allele, and 22 possessed at least one of the XL allele.

Figure 6 gives the relationship between genotype and expression level in female FACHS subjects. Ordinal regression analysis using a simple 0 (SS), 1 (LL) and 2 (at least one XL allele) model demonstrated a significant relationship between genotype and gene expression ($p<0.05$) with the order of transcriptional efficiency being XL > L > S. This pattern is consistent with the hypothesis that the XL variant will be associated with

increased transcriptional efficiency. It is also noteworthy that the relative transcriptional efficiency of the L and S alleles is in the same direction in this African American sample as was reported earlier by our lab and others for an European American sample [61]. Accordingly, there is support for a simple model in which length of the polymorphic region is linearly associated with transcriptional activity, but no support for the hypothesis that regulation of the highly conserved serotonin transmitter system is differentially regulated by African Americans and European Americans. Conversely, because the XL allele is present, there is potential for the 5HTTLPR to provide an expanded range of potential mRNA responses and environmental interactions in gene-environment interaction studies in African American cohorts.

Human serotonin transporter gene expression normalization with multiple housekeeping genes

Gene expression analysis has become an increasingly trusted method in medical research for exploring and understanding complex regulatory networks in diseases. Gene expression patterns allow researchers to further deduce the architecture of gene-environmental interactions affecting certain phenotypic outcomes. RT-PCR is a widely used, highly specific, reproducible and sensitive method for accurately quantifying gene expression of genes under numerous pathological conditions. Although this high-throughput approach is commonly used, scant attention is often given to the use of internal standardization or "control genes" which are vital for compensating well-to-well variability in total RNA levels. These control genes are referred to as housekeeping genes (HKGs) and must have stable gene expression patterns in the tissue or cell or experimental treatment that is being used [93]. In an attempt to determine the appropriate number of HKGs for normalizing the gene expression of the SLC6A4 gene derived for lymphoblast cell lines, a geometric mean based analysis of multiple HKGs was performed. The analysis was performed with the hypothesis that increasing number of

HKGs would increase the strength of association between samples and gene expression with a higher R-squared (R^2) value in a linear regression.

Five commonly used housekeeping genes that were included in the analysis were the calreticulin (CALR), ribosomal protein L7a (RPL7A), ribosomal protein S19 (RPS19), ribosomal protein S20 (RPS20), and ubiquitin C (UBC) genes. The gene expression of the SLC6A4 splice variant 1A+1B and the total gene expression of the SLC6A4 gene (exon 8) were normalized against all combinations of the HKGs ranging from one through all five genes. The transcriptional profiles of the HKGs and the target genes are depicted in Figure 7 and 8 respectively. Lower C_T values represent higher gene expression and vice versa.

In order to normalize the gene expression of exons 1A+1B and 8 with the HKGs, a computer code was written on Python Version 2.7 (Python Software Foundation, USA) to compute the geometric means of the HKGs in all possible combinations. The gene expression values of exons 1A+1B and 8 were then corrected with respect to the calculated geometric means based on specific combinations, and a linear regression was consequently performed to obtain the R^2 values. The largest R^2 value (largest association, lowest variation) from each combination (one through five) were plotted on a histogram for exons 1A+1B and 8 as shown in Figures 9 and 10 respectively.

Although it was hypothesized that increasing number of HKGs would result in higher R^2 values, this was not particularly apparent from Figures 9 and 10. In contrary, the values decreased with an increasing number of HKGs. Nevertheless, the association strength between samples and the gene expression of exon 8 decreased lesser with the addition of HKGs as opposed to exon 1A+1B.

In assessing the normalization of exon 8 specifically, the R^2 values for the HKGs were: UBC > RPL7A > RPS19 > RPS20 > CALR. When the normalization was conducted in a pairwise manner of all possible 10 combinations, the combination of UBC and RPL7A gave the strongest association. Similarly, the analysis performed with three

HKGs in all possible 10 combinations, coupling UBC, RPL7A and RPS19 provided the best result. This pattern was also valid when four HKGs in all possible 5 combinations were assessed. Even though the normalization of exon 1A+1B gene expression did not exhibit this exact pattern, it was almost identical.

From the analysis, although utilizing only one HKG seems adequate, this could be potentially misleading. Furthermore, there are studies that have demonstrated that a relatively high normalization error is present when performing normalization with only one internal control [93]. Therefore, it is highly encouraged to implement the practice of normalizing gene expression data with a panel of HKGs for much accurate transcriptional quantification. However, it is important to note that validating these internal controls under intended experimental conditions prior to conducting RT-PCR and normalizing the gene expression of target genes would help prevent erroneous results. This would also ensure that the best possible HKGs are recommended for future studies enabling data to be compared between studies. To the best of our knowledge, this is the first study exploring several HKG normalization of gene expression data derived from human derived lymphoblast cell lines. Therefore, a larger panel of HKGs is required to be investigated before recommending several HKGs for normalizing human lymphoblast cell lines gene expression data.

Table 1. Clinical, demographic and genetic characteristics of 158 IAS subjects

	History of Abuse	No History of Abuse
N	26	132
Age	45 ± 7	46 ± 8
Sexual Abuse		
Family Member Only	13	
Non-Family Member	10	
Both	3	
Ethnicity		
White	25	124
AA	0	2
White of Hispanic Origin	0	3
Other	1	1
Genotype		
SS	3	26
SL	9	62
LL	14	44

*Total ethnicity does not add up to 158 because the ethnicity of 2 individuals were not available

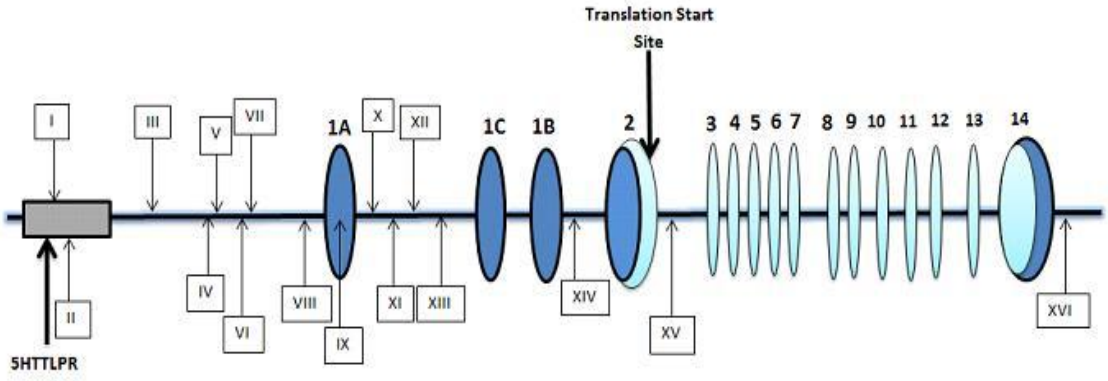

Figure 5. The position of the methylation probes relative to the structure of SLC6A4. Numbers in bold represents the 14 exons present in this gene. Dark blue ovals correspond to untranslated regions (UTR) of SLC6A4 while light blue ovals correspond to translated portions of the exons with the translation start site being contained within exon 2. The "*first exon*" actually contains three variably splice DNA segments labeled 1A, 1C and 1B. The identity of the methylation probes is indicated by boxes with Roman numerals. Probes I (cg1207449), II (cg06841846), and III (cg18584905) are situated at the "south shore" of the gene upstream of the transcription start site (TSS). Probes IV (cg27569822), V (cg10901968), VI (cg26741280), VII (cg25725890) and VIII (cg05016953) are contained within the portion of the CpG island proper upstream of the transcription start site (TSS) while Probe IX (cg14692377) is contained both within the CpG island proper and exon 1A. Probes X (cg03363743), XI (cg22584138) and XII (cg05951817) are situated at the north shore and Probe XIII (cg26126367) at the north shelf of the gene in intron 1A. Probe XIV (cg01330016) is in intron 1B while Probe XV (cg24984698) intron 2. Finally, Probe XVI (cg20592995) is immediately distal to the 3'UTR. The transcription start site is at exon 1A.

Table 2. Relationship between genotype and gene expression of 158 IAS subjects

Genotype	N	Exon 1 Z Score	Exon 8 Z Score
SS	29	-0.053 ± 0.84	-0.387 ± 1.45
SL	71	-0.004 ± 0.90	0.043 ± 0.83
LL	58	-0.182 ± 0.98	0.012 ± 0.91

Table 3. DNA methylation probe characteristics and their relationship to gene expression of 158 IAS subjects

Probe	ID	TSS Position	Island Status	Average β value	Exon 1A+1B p-value	Exon 8 p-value
I	cg12074493	-1416	S Shore	0.09	0.943	0.430
II	cg06841846	-1393	S Shore	0.09	0.809	0.982
III	cg18584905	-599	S Shore	0.20	0.666	0.634
IV	cg27569822	-466	Island	0.04	0.251	0.623
V	cg10901968	-455	Island	0.05	0.708	0.719
VI	cg26741280	-436	Island	0.15	0.670	0.796
VII	cg25725890	-353	Island	0.10	0.113	0.031*
VIII	cg05016953	-112	Island	0.09	0.205	0.056
IX	cg14692377	22	Island	0.19	0.119	0.516
X	cg03363743	227	Island	0.30	0.952	0.594
XI	cg22584138	433	N Shore	0.41	0.041*	0.779
XII	cg05951817	511	N Shore	0.39	0.009*	0.716
XIII	cg26126367	3204	N Shelf	0.81	0.966	0.725
XIV	cg01330016	12315		0.78	0.572	0.459
XV	cg24984698	14157		0.62	0.139	0.573
XVI	cg20592995	38541		0.59	0.485	0.478

Table 4. Effects of genotype and childhood sexual abuse on DNA methylation[1]

Probe	Genotype Significance	Abuse Significance	Genotype x Abuse Significance
cg12074493	0.889	0.786	0.498
cg06841846	0.200	0.626	0.551
cg18584905	0.008*	0.024*	0.003*
cg27569822	0.818	0.697	0.608
cg10901968	0.760	0.733	0.429
cg26741280	0.347	0.36	0.980
cg25725890	0.841	0.543	0.753
cg05016953	0.556	0.473	0.246
cg14692377	0.735	0.690	0.932
cg03363743	0.634	0.671	0.784
cg22584138	0.042*	0.057	0.080
cg05951817	0.135	0.016*	0.191
cg26126367	0.826	0.337	0.619
cg01330016	0.538	0.294	0.381
cg24984698	0.081	0.212	0.058
cg20592995	0.042*	0.331	0.156
EXON 8	0.762	0.594	0.996
EXON 1	0.417	0.371	0.579

[1] We found a similar pattern of significant effects using all sex abuse prior to age 16, including that by non-family members, but the effects were slightly weaker. In particular there was a significant effect of all sex abuse on methylation at cg05951817

Table 5. Clinical, demographic and genetic characteristics of 139 IAS subjects

	No History of Depression	History of Depression
N	52	87
Age	47 ± 8	46 ± 8
Ethnicity		
White	48	83
AA	1	1
White of Hispanic Origin	3	0
Other	0	2
Genotype		
SS	10	12
SL	28	37
LL	14	38

*Total ethnicity does not add up to 139 because the ethnicity of 1 individual was not available

Table 6. Relationship between genotype and gene expression of 139 IAS subjects

Genotype	N	Exon 1 Z Score	Exon 8 Z Score
SS	22	0.066 ± 0.82	-0.111 ± 1.39
SL	65	-0.010 ± 0.92	-0.033 ± 0.74
LL	52	-0.160 ± 0.87	0.042 ± 0.84

Table 7. DNA methylation probe characteristics and their relationship to gene expression of 139 IAS subjects

Probe	ID	TSS Position	Island Status	Average β value	Exon 1A+1B p-value	Exon 8 p-value
I	cg12074493	-1416	S Shore	0.10	0.852	0.566
II	cg06841846	-1393	S Shore	0.09	0.698	0.706
III	cg18584905	-599	S Shore	0.20	0.714	0.535
IV	cg27569822	-466	Island	0.04	0.231	0.789
V	cg10901968	-455	Island	0.05	0.500	0.596
VI	cg26741280	-436	Island	0.15	1.000	0.832
VII	cg25725890	-353	Island	0.10	0.200	0.043*
VIII	cg05016953	-112	Island	0.10	0.218	0.048*
IX	cg14692377	22	Island	0.19	0.288	0.418
X	cg03363743	227	Island	0.29	0.904	0.439
XI	cg22584138	433	N Shore	0.40	0.035*	0.187
XII	cg05951817	511	N Shore	0.38	0.003*	0.180
XIII	cg26126367	3204	N Shelf	0.81	0.589	0.556
XIV	cg01330016	12315		0.77	0.438	0.784
XV	cg24984698	14157		0.61	0.162	0.792
XVI	cg20592995	38541		0.58	0.444	0.882

Table 8. Effects of genotype and maximum count of depressive symptoms (MD_Max) on DNA methylation

Probe	Genotype Significance	MD_Max Significance	Genotype x MD_Max Significance
cg12074493	0.631	0.751	0.681
cg06841846	0.173	0.466	0.636
cg18584905	0.960	0.589	0.562
cg27569822	0.436	0.860	0.429
cg10901968	0.011*	0.157	0.270
cg26741280	0.232	0.373	0.140
cg25725890	0.119	0.007*	0.017*
cg05016953	0.713	0.376	0.742
cg14692377	0.966	0.333	0.283
cg03363743	0.249	0.585	0.834
cg22584138	0.653	0.942	0.430
cg05951817	0.661	0.598	0.999
cg26126367	0.446	0.417	0.576
cg01330016	0.226	0.870	0.108
cg24984698	0.364	0.060	0.504
cg20592995	0.732	0.045*	0.008*
EXON 8	0.801	0.057	0.372
EXON 1	0.399	0.898	0.255

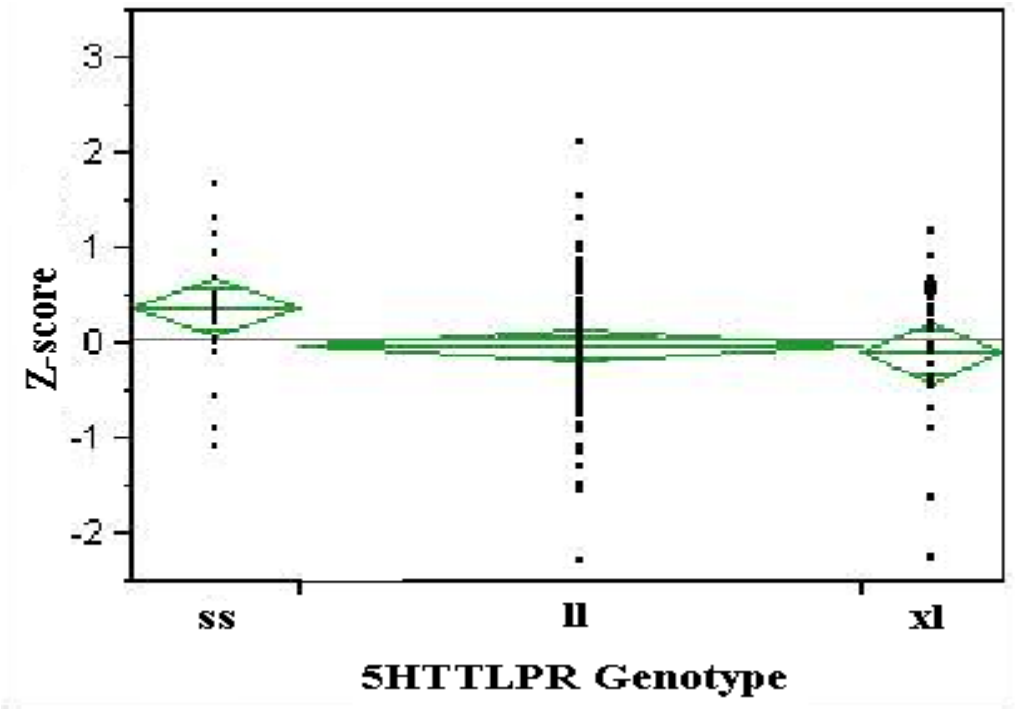

Figure 6. The relationship between 5HTTLPR genotype and SLC6A4 transcript levels in lymphoblast RNA prepared from blood contributed by female FACHS subjects. Higher z-scores denote lower levels of expression with each unit of z-score being equal to approximately 1.6 C_T counts. Group sizes: SS (n=26), LL (n=86) and at least one XL allele (n=22).

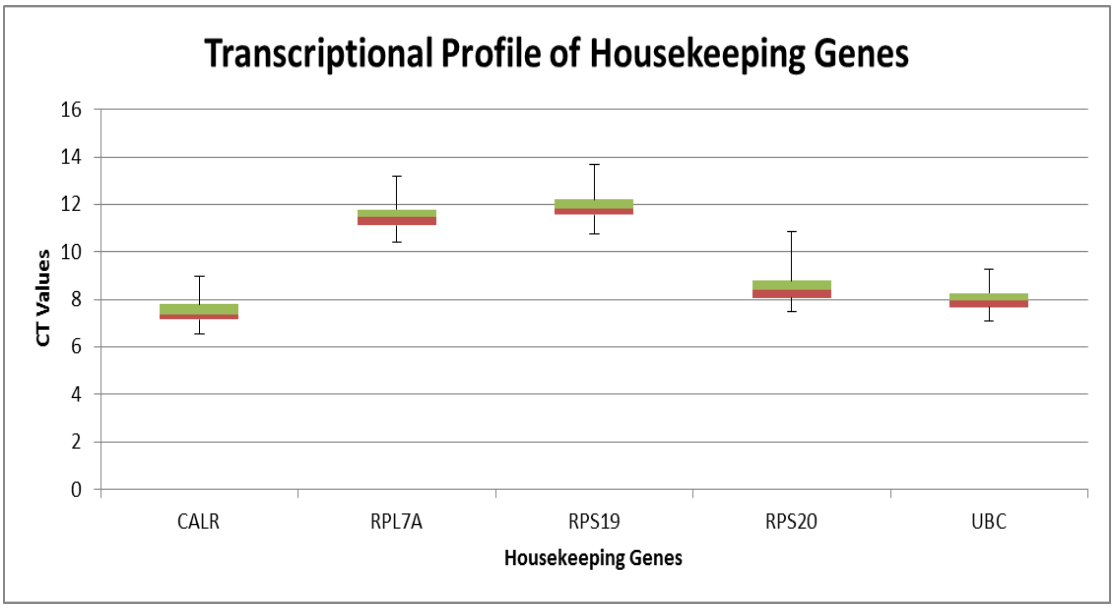

Figure 7. Transcriptional profile of the five housekeeping genes used to normalize the target genes. The housekeeping genes are CALR, RPL7A, RPS19, RPS20, and UBC. Larger C_T values represent lower expression and vice versa.

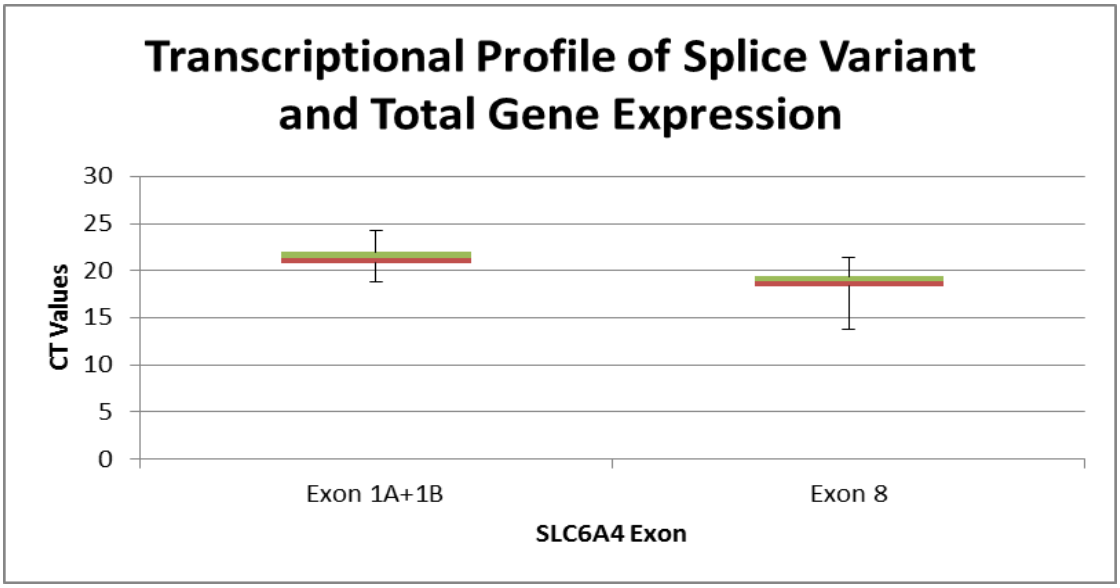

Figure 8. Transcriptional profile of splice variant exon 1A+1B and total (exon 8) gene expression of the SLC6A4 gene. Larger C_T values represent lower expression and vice versa.

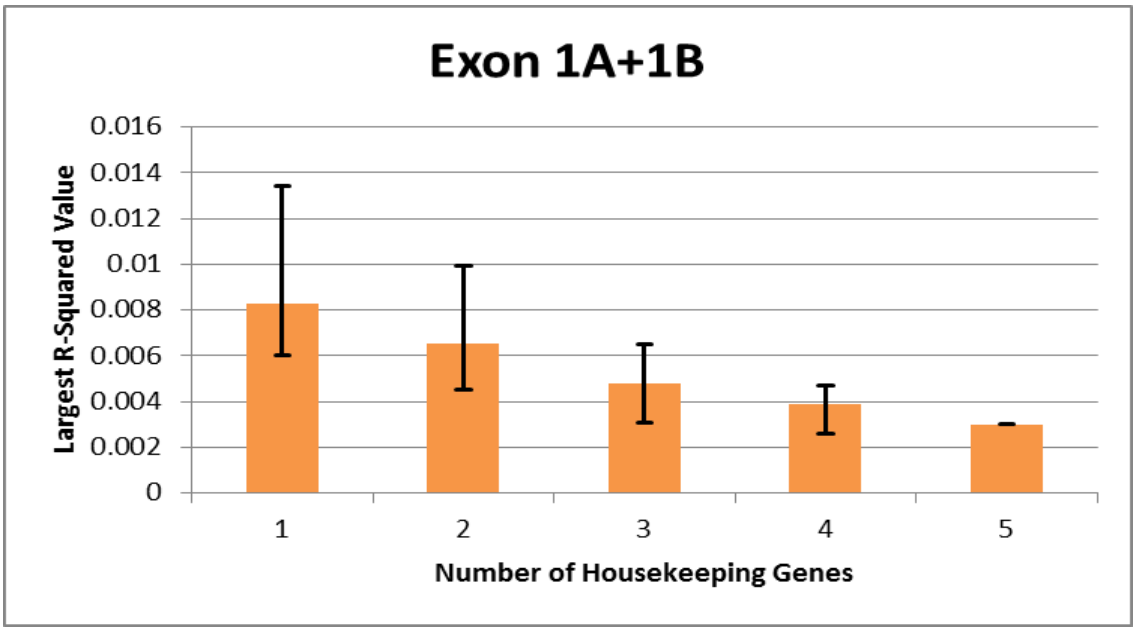

Figure 9. Largest R-squared values obtained from the linear regression of the normalized gene expression of exon 1A+1B with respect to the number of housekeeping gene

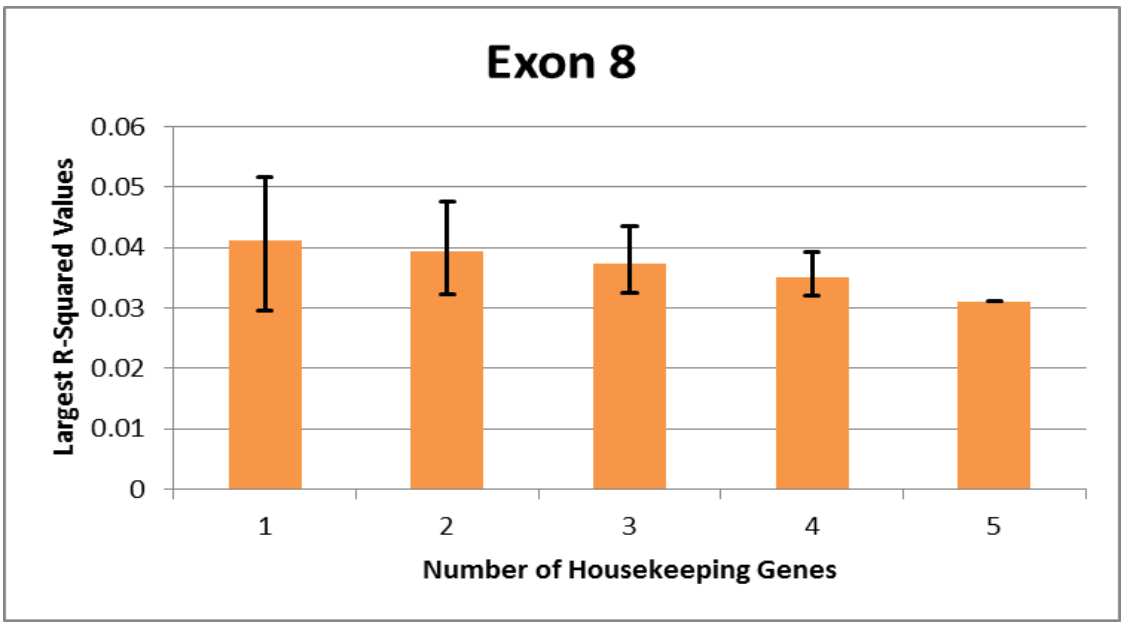

Figure 10. Largest R-squared values obtained from the linear regression of the normalized gene expression of exon 8 with respect to the number of housekeeping gene

CHAPTER 4. SUMMARY AND FUTURE DIRECTIONS

The underlying aim of this project was to further explore aspects related to the mRNA expression of the human serotonin transporter. As mentioned previously, investigations centered on gene expression of a gene are crucial as mRNA expression is intrinsically related to the amount of protein. Therefore, gene expression patterns allow further insight into complex regulatory networks of diseases in an attempt to engineer more personalized and effective therapeutic interventions.

Since the serotonin transporter has been associated with numerous disorders, this locus is an excellent candidate for further exploring genetic, environmental and epigenetic interaction factors to phenotypic outcomes. In this study specifically, the interaction effects of genotype and childhood sexual abuse, and genotype and depression on DNA methylation were investigated. While continuously validating the presence of gene-environmental interactions, we also demonstrated the presence of interaction effects at both instances.

As aforementioned, gene expression of the serotonin transporter has also been shown to be dependent on the 5HTTLPR genotype and vary among populations. In this study, a unique and less explored genetic variant of the 5HTTLPR referred to as the extra-long variant (XL), present predominantly in the African American population was investigated. It was demonstrated that this variant is associated with a higher transcriptional efficiency when compared to the long and short variants.

Finally, since gene expression studies have been expanding rapidly, the normalization of exon 1A+1B and exon 8 gene expressions in the presence of multiple housekeeping genes were examined. Although the results of the examination leaned toward utilizing only one housekeeping gene, it was highly recommended and concluded that more than one housekeeping gene should be used and further investigation is necessary in this area.

Further research is certainly necessary to expand this study and validate the reproducibility of the data. Firstly, sample sizes for both childhood sexual abuse and depression analyses can be expanded to determine if demonstrated effects still follow. Furthermore, if methylation of neuronal cells under a specific condition, childhood sexual abuse for instance, can be obtained, this will enable a comparison to be made to the methylation pattern in a lymphoblast cell line under the same condition. Comparison of these two methylation profiles would help determine if the methylation pattern in the CNS is actually translated to that in peripheral blood. Finally, it would be beneficial to construct a reliable panel of housekeeping genes through validation studies specifically for the normalization of gene expression derived from lymphoblast cell lines.

REFERENCES

1. McCann, U.D., et al., *Brain serotonin neurotoxicity and primary pulmonary hypertension from fenfluramine and dexfenfluramine - A systematic review of the evidence.* Jama-Journal of the American Medical Association, 1997. **278**(8): p. 666-672.

2. Curzon, G., *Serotonin and appetite.* Annals of the New York Academy of Sciences, 1990. **600**: p. 521-30; discussion 530-1.

3. McEntee, W.J. and T.H. Crook, *Serotonin, memory, and the aging brain.* Psychopharmacology, 1991. **103**(2): p. 143-9.

4. Young, S.N. and M. Leyton, *The role of serotonin in human mood and social interaction. Insight from altered tryptophan levels.* Pharmacology, biochemistry, and behavior, 2002. **71**(4): p. 857-65.

5. Altamura, C., et al., *Altered neocortical cell density and layer thickness in serotonin transporter knockout mice: a quantitation study.* Cerebral cortex, 2007. **17**(6): p. 1394-401.

6. Lieben, C.K., et al., *Acute tryptophan depletion induced by a gelatin-based mixture impairs object memory but not affective behavior and spatial learning in the rat.* Behavioural brain research, 2004. **151**(1-2): p. 53-64.

7. Murphy, D.L., et al., *Obsessive-compulsive disorder as a 5-HT subsystem-related behavioural disorder.* The British journal of psychiatry. Supplement, 1989(8): p. 15-24.

8. Vincent, S.R., *Histochemical localization of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine oxidation in the mouse brain.* Neuroscience, 1989. **28**(1): p. 189-99.

9. Ramamoorthy, S., et al., *Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization.* Proceedings of the National Academy of Sciences of the United States of America, 1993. **90**(6): p. 2542-6.

10. Cumming, P. and A. Gjedde, *Kinetics of the uptake of [3H]paroxetine in the rat brain.* Synapse, 1993. **15**(2): p. 124-9.

11. Frankle, W.G., et al., *Estimation of serotonin transporter parameters with 11C-DASB in healthy humans: reproducibility and comparison of methods.* Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 2006. **47**(5): p. 815-26.

12. Houle, S., et al., *Imaging the serotonin transporter with positron emission tomography: initial human studies with [11C]DAPP and [11C]DASB.* European journal of nuclear medicine, 2000. **27**(11): p. 1719-22.

13. Murphy, D.L., et al., *Serotonin transporter: gene, genetic disorders, and pharmacogenetics.* Molecular interventions, 2004. **4**(2): p. 109-23.

14. Kristensen, A.S., et al., *SLC6 neurotransmitter transporters: structure, function, and regulation.* Pharmacological reviews, 2011. **63**(3): p. 585-640.

15. Gu, H., S.C. Wall, and G. Rudnick, *Stable expression of biogenic amine transporters reveals differences in inhibitor sensitivity, kinetics, and ion dependence.* The Journal of biological chemistry, 1994. **269**(10): p. 7124-30.

16. Torres, G.E., R.R. Gainetdinov, and M.G. Caron, *Plasma membrane monoamine transporters: structure, regulation and function.* Nature reviews. Neuroscience, 2003. **4**(1): p. 13-25.

17. Ozsarac, N., E. Santha, and B.J. Hoffman, *Alternative non-coding exons support serotonin transporter mRNA expression in the brain and gut.* Journal of neurochemistry, 2002. **82**(2): p. 336-44.

18. Colucci, R., et al., *The genetics of the serotonin transporter and irritable bowel syndrome.* Trends in molecular medicine, 2008. **14**(7): p. 295-304.

19. Bengel, D., et al., *Gene structure and 5'-flanking regulatory region of the murine serotonin transporter.* Brain research. Molecular brain research, 1997. **44**(2): p. 286-92.

20. Bradley, C.C. and R.D. Blakely, *Alternative splicing of the human serotonin transporter gene.* Journal of neurochemistry, 1997. **69**(4): p. 1356-67.

21. Heils, A., et al., *Functional promoter and polyadenylation site mapping of the human serotonin (5-HT) transporter gene.* Journal of neural transmission. General section, 1995. **102**(3): p. 247-54.

22. Heils, A., et al., *Allelic variation of human serotonin transporter gene expression.* Journal of neurochemistry, 1996. **66**(6): p. 2621-4.

23. Lesch, K.P., et al., *Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region.* Science, 1996. **274**(5292): p. 1527-31.

24. Hu, X., et al., *An expanded evaluation of the relationship of four alleles to the level of response to alcohol and the alcoholism risk.* Alcoholism, clinical and experimental research, 2005. **29**(1): p. 8-16.

25. Hu, X.Z., et al., *Serotonin transporter promoter gain-of-function genotypes are linked to obsessive-compulsive disorder.* American journal of human genetics, 2006. **78**(5): p. 815-26.

26. Kraft, J.B., et al., *Sequence analysis of the serotonin transporter and associations with antidepressant response.* Biological psychiatry, 2005. **58**(5): p. 374-81.

27. Nakamura, M., et al., *The human serotonin transporter gene linked polymorphism (5-HTTLPR) shows ten novel allelic variants.* Molecular psychiatry, 2000. **5**(1): p. 32-8.

28. Wendland, J.R., et al., *Simultaneous genotyping of four functional loci of human SLC6A4, with a reappraisal of 5-HTTLPR and rs25531.* Molecular psychiatry, 2006. **11**(3): p. 224-6.

29. Wray, N.R., et al., *Accurate, Large-Scale Genotyping of 5HTTLPR and Flanking Single Nucleotide Polymorphisms in an Association Study of Depression, Anxiety, and Personality Measures.* Biological psychiatry, 2009. **66**(5): p. 468-76.

30. Serretti, A., et al., *Meta-analysis of serotonin transporter gene promoter polymorphism (5-HTTLPR) association with selective serotonin reuptake inhibitor efficacy in depressed patients.* Molecular psychiatry, 2007. **12**(3): p. 247-57.

31. Smits, K.M., et al., *Influence of SERTPR and STin2 in the serotonin transporter gene on the effect of selective serotonin reuptake inhibitors in depression: a systematic review.* Molecular psychiatry, 2004. **9**(5): p. 433-41.

32. Mortensen, O.V., et al., *Functional analysis of a novel human serotonin transporter gene promoter in immortalized raphe cells.* Brain research. Molecular brain research, 1999. **68**(1-2): p. 141-8.

33. Kaiser, R., et al., *Correlation between serotonin uptake in human blood platelets with the 44-bp polymorphism and the 17-bp variable number of tandem repeat of the serotonin transporter.* American journal of medical genetics, 2002. **114**(3): p. 323-8.

34. Delbruck, S.J., et al., *A novel allelic variant of the human serotonin transporter gene regulatory polymorphism.* Cytogenetics and cell genetics, 1997. **79**(3-4): p. 214-20.

35. Hranilovic, D., et al., *Serotonin transporter promoter and intron 2 polymorphisms: relationship between allelic variants and gene expression.* Biological psychiatry, 2004. **55**(11): p. 1090-4.

36. Kessler, R.C., et al., *The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R).* JAMA : the journal of the American Medical Association, 2003. **289**(23): p. 3095-105.

37. Paulus, M.P. and M.B. Stein, *Interoception in anxiety and depression.* Brain structure & function, 2010. **214**(5-6): p. 451-63.

38. Kessler, R.C., et al., *Sex and depression in the National Comorbidity Survey. I: Lifetime prevalence, chronicity and recurrence.* Journal of affective disorders, 1993. **29**(2-3): p. 85-96.

39. Murray, C.J. and A.D. Lopez, *Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study.* Lancet, 1997. **349**(9063): p. 1436-42.

40. Greenberg, P.E., et al., *The economic burden of depression in the United States: how did it change between 1990 and 2000?* The Journal of clinical psychiatry, 2003. **64**(12): p. 1465-75.

41. Owens, M.J. and C.B. Nemeroff, *Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter.* Clinical chemistry, 1994. **40**(2): p. 288-95.

42. Roy, A., J. De Jong, and M. Linnoila, *Cerebrospinal fluid monoamine metabolites and suicidal behavior in depressed patients. A 5-year follow-up study.* Archives of general psychiatry, 1989. **46**(7): p. 609-12.

43. Meyer, J.H., et al., *Brain serotonin transporter binding potential measured with carbon 11-labeled DASB positron emission tomography: effects of major depressive episodes and severity of dysfunctional attitudes.* Archives of general psychiatry, 2004. **61**(12): p. 1271-9.

44. Kendler, K.S., et al., *Stressful life events, genetic liability, and onset of an episode of major depression in women.* The American journal of psychiatry, 1995. **152**(6): p. 833-42.

45. Caspi, A., et al., *Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene.* Science, 2003. **301**(5631): p. 386-9.

46. Brown, G.W. and T.O. Harris, *Depression and the serotonin transporter 5-HTTLPR polymorphism: A review and a hypothesis concerning gene-environment interaction.* Journal of affective disorders, 2008. **111**(1): p. 1-12.

47. Kendler, K.S., et al., *The interaction of stressful life events and a serotonin transporter polymorphism in the prediction of episodes of major depression: a replication.* Archives of general psychiatry, 2005. **62**(5): p. 529-35.

48. Eley, T.C., et al., *Gene-environment interaction analysis of serotonin system markers with adolescent depression.* Molecular psychiatry, 2004. **9**(10): p. 908-15.

49. Kim, J.M., et al., *Interactions between life stressors and susceptibility genes (5-HTTLPR and BDNF) on depression in Korean elders.* Biological psychiatry, 2007. **62**(5): p. 423-8.

50. Wilhelm, K., et al., *Life events, first depression onset and the serotonin transporter gene.* The British journal of psychiatry : the journal of mental science, 2006. **188**: p. 210-5.

51. Cervilla, J.A., et al., *The risk for depression conferred by stressful life events is modified by variation at the serotonin transporter 5HTTLPR genotype: evidence from the Spanish PREDICT-Gene cohort.* Molecular psychiatry, 2007. **12**(8): p. 748-55.

52. Dick, D.M., et al., *Association analyses of the serotonin transporter gene with lifetime depression and alcohol dependence in the Collaborative Study on the Genetics of Alcoholism (COGA) sample.* Psychiatric genetics, 2007. **17**(1): p. 35-8.

53. Champoux, M., et al., *Serotonin transporter gene polymorphism, differential early rearing, and behavior in rhesus monkey neonates.* Molecular psychiatry, 2002. **7**(10): p. 1058-63.

54. Lesch, K.P., et al., *The 5-HT transporter gene-linked polymorphic region (5-HTTLPR) in evolutionary perspective: alternative biallelic variation in rhesus monkeys. Rapid communication.* Journal of neural transmission, 1997. **104**(11-12): p. 1259-66.

55. Bennett, A.J., et al., *Early experience and serotonin transporter gene variation interact to influence primate CNS function.* Molecular psychiatry, 2002. **7**(1): p. 118-22.

56. Henikoff, S. and M.A. Matzke, *Exploring and explaining epigenetic effects.* Trends in genetics : TIG, 1997. **13**(8): p. 293-5.

57. Jaenisch, R. and A. Bird, *Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals.* Nature genetics, 2003. **33 Suppl**: p. 245-54.

58. Mill, J. and A. Petronis, *Molecular studies of major depressive disorder: the epigenetic perspective.* Molecular psychiatry, 2007. **12**(9): p. 799-814.

59. Liu, L., Y. Li, and T.O. Tollefsbol, *Gene-environment interactions and epigenetic basis of human diseases.* Current issues in molecular biology, 2008. **10**(1-2): p. 25-36.

60. Bird, A., *DNA methylation patterns and epigenetic memory.* Genes & development, 2002. **16**(1): p. 6-21.

61. Philibert, R.A., et al., *The relationship of 5HTT (SLC6A4) methylation and genotype on mRNA expression and liability to major depression and alcohol dependence in subjects from the Iowa Adoption Studies.* American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics, 2008. **147B**(5): p. 543-9.

62. van, I.M.H., et al., *Methylation matters: interaction between methylation density and serotonin transporter genotype predicts unresolved loss or trauma.* Biological psychiatry, 2010. **68**(5): p. 405-7.

63. Crittenden, P.M., *Abusing, Neglecting, Problematic, and Adequate Dyads - Differentiating by Patterns of Interaction.* Merrill-Palmer Quarterly-Journal of Developmental Psychology, 1981. **27**(3): p. 201-218.

64. Erickson, M.F. and B. Egeland, *A Developmental View of the Psychological Consequences of Maltreatment.* School Psychology Review, 1987. **16**(2): p. 156-168.

65. Trickett, P.K. and C. Mcbridechang, *The Developmental Impact of Different Forms of Child-Abuse and Neglect.* Developmental Review, 1995. **15**(3): p. 311-337.

66. Dodge, K.A., J.E. Bates, and G.S. Pettit, *Mechanisms in the Cycle of Violence.* Science, 1990. **250**(4988): p. 1678-1683.

67. Hoffmanplotkin, D. and C.T. Twentyman, *A Multimodal Assessment of Behavioral and Cognitive Deficits in Abused and Neglected Preschoolers.* Child Development, 1984. **55**(3): p. 794-802.

68. Trickett, P.K. and F.W. Putnam, *Impact of Child Sexual Abuse on Females - toward a Developmental, Psychobiological Integration.* Psychological Science, 1993. **4**(2): p. 81-87.

69. Trickett, P.K., et al., *Relationship of Socioeconomic-Status to the Etiology and Developmental Sequelae of Physical Child-Abuse.* Developmental Psychology, 1991. **27**(1): p. 148-158.

70. White, S., et al., *Behavioral-Comparisons of Young Sexually Abused, Neglected, and Nonreferred Children.* Journal of Clinical Child Psychology, 1988. **17**(1): p. 53-61.

71. Friedrich, W.N., A.J. Urquiza, and R.L. Beilke, *Behavior problems in sexually abused young children.* Journal of pediatric psychology, 1986. **11**(1): p. 47-57.

72. Fromuth, M.E., *The relationship of childhood sexual abuse with later psychological and sexual adjustment in a sample of college women.* Child Abuse & Neglect, 1986. **10**(1): p. 5-15.

73. Murphy, G.E., *Suicide and substance abuse.* Archives of general psychiatry, 1988. **45**(6): p. 593-4.

74. Sedney, M.A. and B. Brooks, *Factors associated with a history of childhood sexual experience in a nonclinical female population.* Journal of the American Academy of Child Psychiatry, 1984. **23**(2): p. 215-8.

75. Mullen, P.E., et al., *Impact of sexual and physical abuse on women's mental health.* Lancet, 1988. **1**(8590): p. 841-5.

76. Cicchetti, D. and D. Tucker, *Development and Self-Regulatory Structures of the Mind.* Development and Psychopathology, 1994. **6**(4): p. 533-549.

77. Spoont, M.R., *Modulatory role of serotonin in neural information processing: implications for human psychopathology.* Psychological bulletin, 1992. **112**(2): p. 330-50.

78. Kaufman, J., et al., *Social supports and serotonin transporter gene moderate depression in maltreated children.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(49): p. 17316-17321.

79. Beach, S.R., et al., *Methylation at SLC6A4 is linked to family history of child abuse: an examination of the Iowa Adoptee sample.* American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics, 2010. **153B**(2): p. 710-3.

80. Beach, S.R., et al., *Methylation at 5HTT mediates the impact of child sex abuse on women's antisocial behavior: an examination of the Iowa adoptee sample.* Psychosomatic medicine, 2011. **73**(1): p. 83-7.

81. Philibert, R., *Merging genetic and environmental effects in the Iowa Adoption Studies: focus on depression.* Annals of clinical psychiatry : official journal of the American Academy of Clinical Psychiatrists, 2006. **18**(4): p. 219-22.

82. Guyll, M., et al., *Hostility, relationship quality, and health among African American couples.* Journal of Consulting and Clinical Psychology, 2010. **78**(5): p. 646-54.

83. Bradley, S.L., et al., *Relationship of serotonin transporter gene polymorphisms and haplotypes to mRNA transcription.* American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics, 2005. **136B**(1): p. 58-61.

84. Schwanhausser, B., et al., *Global quantification of mammalian gene expression control.* Nature, 2011. **473**(7347): p. 337-42.

85. Monick, M.M., et al., *Coordinated changes in AHRR methylation in lymphoblasts and pulmonary macrophages from smokers.* American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics, 2012. **159B**(2): p. 141-51.

86. Fan, S. and X. Zhang, *CpG island methylation pattern in different human tissues and its correlation with gene expression.* Biochemical and biophysical research communications, 2009. **383**(4): p. 421-5.

87. Philibert, R., et al., *Serotonin transporter mRNA levels are associated with the methylation of an upstream CpG island.* American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics, 2007. **144B**(1): p. 101-5.

88. Gorey, K.M. and D.R. Leslie, *The prevalence of child sexual abuse: integrative review adjustment for potential response and measurement biases.* Child Abuse & Neglect, 1997. **21**(4): p. 391-8.

89. Bulik, C.M., C.A. Prescott, and K.S. Kendler, *Features of childhood sexual abuse and the development of psychiatric and substance use disorders.* The British journal of psychiatry : the journal of mental science, 2001. **179**: p. 444-9.

90. Karg, K., et al., *The serotonin transporter promoter variant (5-HTTLPR), stress, and depression meta-analysis revisited: evidence of genetic moderation.* Archives of general psychiatry, 2011. **68**(5): p. 444-54.

91. Propper, C., et al., *Parenting quality, DRD4, and the prediction of externalizing and internalizing behaviors in early childhood.* Developmental psychobiology, 2007. **49**(6): p. 619-32.

92. Widom, C.S. and L.M. Brzustowicz, *MAOA and the "cycle of violence:" childhood abuse and neglect, MAOA genotype, and risk for violent and antisocial behavior.* Biological psychiatry, 2006. **60**(7): p. 684-9.

93. Vandesompele, J., et al., *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.* Genome biology, 2002. **3**(7): p. RESEARCH0034.