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Rare Variation in the Serotonin Transporter and Autism Spectrum Disorders: Examining 5-HT-mediated Effects on Neurodevelopment and Behavior

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Abstract
Elevated whole blood serotonin (5-HT) levels are present in approximately a quarter of individuals with autism spectrum disorders (ASD). There is mounting evidence that altered developmental 5-HT signaling in the brain may play a role in the etiology of ASD. In particular, the serotonin transporter (SERT), a key regulator of 5-HT homeostasis, has been repeatedly implicated in the disorder. Rare coding variants have been discovered that alter SERT function and regulation, and are associated with specific ASD features. *In vivo* modeling of rare, ASD-associated SERT mutations in mice offers an opportunity to examine how changes in 5-HT signaling during development can disrupt formation of brain circuits and cause specific ASD behavioral symptoms and traits.

Keywords: *Serotonin; Serotonin transporter; Autism spectrum disorder; Restricted, repetitive behavior; Sensory aversion; Gly56Ala; Barrel field; Thalamocortical axons; Mouse model*

ASD Background

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental disorders that share core features of impairment in social communication and interaction, as well as restricted, repetitive behavior. ASD emerges during early childhood, but may not fully manifest itself until social demands are placed on an affected individual. Recent epidemiological studies have estimated the prevalence of ASD, which includes autistic disorder, Asperger syndrome, pervasive developmental disorder not otherwise specified (PDDNOS), and childhood disintegrative disorder (CDD), to be one child in every 150 children[1]. Males are at increased risk for ASD, with an affected male to female ratio of 4:1[1]. In addition to the core diagnostic features, individuals with ASD often display secondary symptoms such as gastrointestinal dysfunction[2], epilepsy[3], and sensory aversion[4] at a much higher rate than the general population.

Among neuropsychiatric disorders, ASD has been shown to be the most heritable[5]. Depending on diagnostic criteria, family and twin studies estimate concordance rates between 60-90% for monozygotic twins compared to 0-10% for dizygotic twins[6]. Parents and siblings of affected individuals are more likely to show subtle behavioral abnormalities that mirror deficits seen in ASD[7,8]. Also, ASD is observed in subpopulations of individuals with rare genetic syndromes, such as fragile X syndrome, Rett syndrome, Angelman syndrome, and tuberous sclerosis[9]. Genetic linkage and association studies have implicated numerous candidate genes in the etiology of ASD. From these studies, functional single nucleotide polymorphisms (SNPs) and copy number variants (CNVs) have been discovered that may underlie the genetic risk. However, it is evident that ASD is a complex, polygenic disorder. To date, no single genetic variation has been implicated in more than 1-2% of ASD cases[9]. This has led some to hypothesize that the additive effects of multiple risk alleles may cause ASD and contribute to the phenotypic heterogeneity of the disorder[10]. Data emerging from genome wide association studies have yet to confirm this hypothesis, but these studies have been statistically underpowered to reliably detect alleles that confer small or moderate risk[11].

Serotonin and ASD

One of the oldest, yet most consistently replicated findings in individuals with ASD is elevated whole blood serotonin (5-hydroxytryptamine, 5-HT) or hyperserotonemia[12]. Studies have estimated that 25% of children with ASD have hyperserotonemia[13]. In the periphery, 5-HT is synthesized by enterochromaffin cells that line the gastrointestinal tract. It is then released into enteric circulation and taken up by platelets, which contain approximately 99% of 5-HT in the blood[14]. Multiple studies have indicated a positive correlation of 5-HT platelet levels between ASD cases and their parents and siblings[15,16]. In the Hutterites, a large founder population, whole blood 5-HT levels were found to be more heritable than ASD itself[17]. Since hyperserotonemia has been shown to be a reliable endophenotype[A] for ASD, there has been a great deal of interest in the genetics and molecular mechanisms that control 5-HT uptake into platelets.

Investigations of disrupted platelet 5-HT regulation in ASD have primarily focused on the integrin β3 subunit (ITGB3), 5-HT_2 receptors, and the serotonin transporter (SERT or 5-HTT). Additional linkage and association studies in the Hutterites mapped *ITGB3* and *SLC6A4*, the gene encoding SERT, as quantitative trait loci for whole blood 5-HT levels[18]. Variation in *ITGB3*[19] and a gene-gene interaction with *SLC6A4*[20] have been associated with ASD susceptibility. Furthermore, there is evidence of decreased platelet 5-HT_2 receptor binding in ASD[21]. Activation of 5-HT_{2A} receptors has been shown to regulate SERT localization in platelets[22], providing a potential mechanism that could be disrupted in hyperserotonemic ASD cases. Due to its central role in the uptake of 5-HT, the serotonin transporter has been the main focus of research into serotonergic function in ASD, and will be discussed at length later in this review.

Ultimately, peripheral 5-HT findings in ASD must be applied to the brain to understand the complex cognitive and behavioral features of the disorder. Mirroring data from platelets, human neuroimaging studies have found decreased brain 5-HT_2 receptor binding in ASD[23,24]. In addition, depletion of the amino acid tryptophan, the precursor of 5-HT, has been shown to exacerbate stereotyped behaviors in adults with ASD[25]. Tryptophan

[A] **Endophenotype**: a behavioral, cognitive, or biological measure that is a heritable trait in a psychiatric disorder.

depletion acutely reduces serotonin synthesis, leading to a hypothetical net reduction in serotonergic neurotransmission. Interestingly, selective serotonin reuptake inhibitors (SSRIs), which have the net effect of increasing serotonergic neurotransmission, have been shown to ameliorate repetitive behavior and aggression in ASD[26,27]. Altered developmental trajectories of serotonin synthesis capacity in the brain have also been reported in the disorder[28]. These converging lines of evidence suggest that 5-HT abnormalities in the brain and the periphery play a role in the etiology of ASD.

SERT and ASD

Due to the replicated finding of hyperserotonemia as an ASD biomarker, SERT has been an attractive research target for the disorder. Similar to other monoamine transporters, SERT is an integral plasma membrane protein composed of twelve transmembrane domains, which actively transports 5-HT in an ion-coupled and anti-depressant sensitive manner[29,30]. SERT terminates 5-HT signaling by transporting 5-HT from the synaptic space back into presynaptic terminals, where the neurotransmitter can be repackaged into vesicles for rerelease[31]. ASD linkage studies have implicated the chromosome 17q11-q21 region that contains *SLC6A4*, the gene that encodes SERT[32,33]. When families with only affected males are considered, the linkage signal in *SLC6A4* region significantly increases, suggesting SERT may harbor variants that play a role in the gender bias in ASD[33,34].

The effect of common genetic variation in *SLC6A4* has been the source of much debate in neuropsychiatric research. In particular, the serotonin transporter gene linked polymorphic region (5-HTTLPR) has been scrutinized for its potential role in ASD. The 5-HTTLPR contains a 44 base pair insertion/deletion polymorphism that has a functional impact on transcription[35]. The short allele, which reduces *SLC6A4* expression, has been overtransmitted in ASD compared to controls in several studies[36,37]. However, this association has not been consistently replicated[38,39], and other functional variants in the gene further complicate 5-HTTLPR analysis. Two SNPs in the promoter region, rs25531[40] and rs25532[41], can modulate activity of the long allele and reduce *SLC6A4* expression. In addition, a variable number of tandem repeats (VNTR) polymorphism in intron 2 has also been shown to regulate *SLC6A4* expression[42]. The inconsistent associations with ASD have led some groups to speculate that common *SLC6A4* variation may modulate specific behavioral features and symptoms of ASD. Brune *et al.* found that the 5-HTTLPR short allele is associated with poor nonverbal communication in ASD, while the long allele is associated with stereotyped, restrictive motor mannerisms and aggressive behavior[43]. Also, Mulder *et al.* reported association between the intron 2 VNTR 12-repeat allele and rigid-compulsive behavior in ASD[44].

Although common genetic variants of SERT have been an area of intense focus, modest association results have not explained the suggestive linkage of the *SLC6A4* locus in ASD. To determine if multiple, rare functional variants contribute towards *SLC6A4* linkage, Sutcliffe *et al.* screened 120 multiplex families that had a significant male biased linkage signal at 17q11[34]. In their sample population, they found five rare non-synonymous coding SNPs that were overtransmitted to affected family members and were associated with rigid-compulsive behavior: Gly56Ala, Ile425Leu, Phe465Leu, Leu550Val, Lys605Asn[34]. Three of the coding variants, Ile425Leu, Phe465Leu, Leu550Val, are located within transmembrane domains at highly conserved amino acids[34]. Interestingly, Ozaki *et al*. discovered a SERT Ile425Val variant in two unrelated OCD pedigrees that had comorbidity for Asperger syndrome and other neuropsychiatric disorders[45]. The Gly56Ala variant, which will be focused upon later in this review, is located at a highly conserved amino acid residue in the intracellular N-terminus tail of SERT and was the most common rare mutation found in the Sutcliffe *et al*. study[34]. The Ala56 allele was overtransmitted 3:1 to individuals with ASD, displayed a male gender bias, and was significantly associated with sensory aversion[B] and rigid-compulsive behavior[34]. Also, initial characterization of transformed lymphoblasts from families with the SERT Ala56 allele indicated that the variant caused elevated 5-HT uptake that was insensitive to protein kinase G (PKG) and p38 mitogen activated protein kinase (p38 MAPK) pathway regulation[34]. The discovery of these rare SERT variants supports the hypothesis that allelic heterogeneity of *SLC6A4* confers risk of ASD.

Serotonin and Neurodevelopment

Although 5-HT is primarily known as a canonical neurotransmitter, there is mounting evidence that it has a pleiotropic[C] role in neurodevelopment. The early maturation of the serotonergic system initially prompted speculation that 5-HT had a developmental function. In the brain, 5-HT is exclusively produced in the rostral and caudal raphe nuclei groups (B1-B9). These serotonergic neurons are produced as early as E10-12 in rodents and begin producing 5-HT within a day of their birth[46]. In addition to endogenously produced 5-HT, maternal 5-HT can cross the placenta and fetal blood-brain barrier[47]. The rostral raphe nuclei groups (B6-B9) consist of serotonergic neurons whose axons project towards the telencephalon. Serotonergic afferents reach the telencephalon at E15, as the cortical plate begins to form[46]. Several 5-HT receptor subtypes have shown embryonic expression in a variety of brain regions[48,49]. In a landmark study demonstrating the developmental role of 5-HT signaling, Gross *et al.* found that temporally restricted deletion of 5-HT_{1A} receptors during the early postnatal period was sufficient to produce anxiety-like phenotypes in adult mice[50].

5-HT mediated neurodevelopmental processes are now beginning to be explored in greater detail. Pharmacological and genetic manipulation of 5-HT levels in the embryonic brain have implicated 5-HT as a modulator of neuronal migration. Vitalis *et al*. demonstrated that embryonic 5-HT depletion with p-chlorophenylalanine (PCPA), an inhibitor of serotonin synthesis, causes migratory and differentiation defects in specific populations of GABAergic interneurons emanating from the caudal ganglionic eminence (CGE)[51]. The authors speculate that 5-HT_3 receptors, which are expressed by CGE-derived interneurons, may regulate migration[51]. In contrast, Riccio *et al.* examined GABAergic interneuron migration defects in SERT knockout mice, which have elevated synaptic 5-HT levels[52]. Interestingly, they found that only interneurons expressing 5-HT_6 receptors were sensitive to excessive 5-HT during development[52]. Furthermore, SERT knockouts displayed altered interneuron density in primary somatosensory cortex[52], a well-documented 5-HT sensitive brain region.

The developmental impact of altered 5-HT levels has been intensely studied in rodent barrel field architecture of primary somatosensory cortex. Barrel field architecture is formed by thalamocortical axons (TCAs) synapsing with layer IV cortical neurons[53]. Each individual barrel is a cortical representation of a mystacial vibrissa , where layer IV neurons within a barrel will fire in response to a tactile stimulus applied to a particular vibrissa[53]. Removal of monoamine oxidase A (MAOA), the main enzyme responsible for 5-HT degradation, has been shown to eliminate barrel field formation due to excessive 5-HT levels during the perinatal period of development[54]. In an effort to understand how excessive 5-HT could disrupt cortical soma-

[B] **Sensory aversion**: hypersensitivity to multiple sensory modalities that causes distress.
[C] **Pleiotropic**: producing more than one effect.

tosensory maps, Salichon *et al*. crossed MAOA knockouts with mice lacking SERT or the 5-HT_{1B} receptor[55], which are both transiently expressed in TCAs during development[56,57]. The authors determined that disruptions in barrel field formation were due to overactivation of 5-HT_{1B} receptors by increased levels of synaptic 5-HT[55].

Recently, Bonnin *et al.* further examined the molecular mechanisms involved in 5-HT regulation of thalamocortical axonal pathfinding[58]. During development, 5-HT_{1B} and 5-HT_{1D} receptors in the thalamus have overlapping expression with axon guidance receptors DCC and Unc5c[58]. Using thalamic explant cultures, they found that attractive cues exerted by netrin-1 on posterior TCAs became repulsive with activation of 5-$HT_{1B/1D}$ receptors[58]. Also, TCAs could be deflected *in vivo* using *in utero* electroporation of siRNAs and expression plasmids to alter 5-HT_{1B} and 5-HT_{1D} receptor expression in the thalamus[58]. Their data suggest that alterations in 5-HT signaling during development could cause subtle topographical shifts in TCA brain circuits. Furthermore, Esaki *et al.* have presented evidence that developmental changes in TCA circuits may have a functional impact in adulthood. They reported that SERT knockout mice, which possess disrupted barrel field architecture, have altered cerebral glucose utilization during whisker stimulation[59]. Perinatal administration of PCPA restored normal somatosensory responses in adult SERT knockouts[59]. Collectively, these studies support the hypothesis that disruptions in the serotonergic system during development could be the basis for a neurodevelopmental disorder such as ASD.

SERT Gly56Ala: Modeling ASD in Mice

As described earlier in this review, a rare SERT coding variant, Gly56Ala variant, is associated with ASD, rigid-compulsive behavior, and sensory aversion in families with evidence of linkage at the *SLC6A4* locus[34]. Characterization of lymphoblasts from ASD probands indicated that the 56Ala variant causes elevated 5-HT transport[34]. 5-HT transport was insensitive to PKG and p38 MAPK stimulation, which regulate SERT surface expression and catalytic activity, respectively[34]. Furthermore, SERT Ala56 was found to be hyperphosphorylated under basal conditions and lacks enhanced phosphorylation via PKG stimulation[60]. Additional functional studies of the Ala56 variant in transiently transfected HeLa cells have demonstrated alterations of protein kinase C (PKC) and protein phosphatase 2A (PP2A) regulation of SERT[61]. Although *in vitro* analysis of ASD-associated SERT variants has greatly aided our understanding of SERT function and regulation, *in vivo* brain studies are now necessary to understand how a given mutation impacts behavior and neurodevelopment. With this goal in mind, our lab generated a knock-in mouse line with the most common ASD-associated SERT variant, the Ala56 allele, inserted into the native mouse 129S6 *Slc6a4* gene[62].

New discoveries of rare variants strongly implicated in the etiology of ASD have spurred the development of mouse models with analogous mutations to evaluate comparable ASD phenotypes and to dissect the molecular mechanisms that underlie ASD traits. In general, effective animal models of human disorders should: 1) exhibit similar symptoms and traits found in the human disorder (face validity); 2) possess the same biological cause as the human disorder (construct validity); 3) respond to treatments that prevent or reverse symptoms of the human disorder (predicative validity)[63]. Due to the complex phenotypes exhibited in ASD, it is challenging for behavioral neuroscientists to design behavioral tasks that examine the face validity of mouse models of ASD.

This review highlights the growing library of assays to examine restricted, repetitive behavior and sensory dysfunction, two traits associated with the SERT Gly56Ala variant in humans. Mice exhibit stereotyped motor behaviors such as circling, jumping, and self-grooming that are altered in models of ASD[64]. Reversal learning tasks, such as T-maze and Morris water maze, measure a mouse's ability to switch from a learned habit to a new habit. Impairments in acquiring a new habit are thought to have face validity to 'insistence of sameness' behavior seen in individuals with ASD[64]. Disruptions in mouse exploratory behavior (i.e. locomotion, sniffing, nose poking) are also being examined as a relevant measure of restricted interests in ASD[65]. Current paradigms that evaluate sensory-related behavior in mice primarily focus on sensorimotor gating or pain tolerance. Deficits in prepulse inhibition (PPI), a measure of sensorimotor gating, have been reported in individuals with ASD[66] and offer a mouse behavioral assay with face validity to an ASD trait. In addition to sensorimotor gating, sensory dysfunction in mice is commonly evaluated by assays that measure nociceptive responses. Behavioral assays such as the hot plate or tail flick test assess thermal sensitivity in mice, but their relevance to ASD symptomology is unclear[67].

Conclusion

The diverse behavioral features and secondary traits that are observed in ASD have largely hindered research into the etiology of the neurodevelopmental disorder. The complex, polygenic origin of ASD is thought to underlie the disorder's phenotypic heterogeneity. Hyperserotonemia, a proven biomarker observed in almost a quarter of ASD cases, offers an avenue of research into the molecular mechanisms and altered brain circuits that produce specific ASD traits. The development of animal models of ASD is necessary for discovering effective treatments. As described in this review, rare variation in SERT has been highly associated with rigid-compulsive disorders in male individuals with ASD. The most common ASD-associated SERT variant, Gly56Ala, is also linked to sensory aversion. *In vivo* modeling of ASD-associated SERT variants offers a unique opportunity to study how altered 5-HT signaling during development can cause specific ASD behavioral features and traits.

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