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ORIGINAL PAPER

Genetic Variation in Melatonin Pathway Enzymes in Children with Autism Spectrum Disorder and Comorbid Sleep Onset Delay

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Abstract Sleep disruption is common in individuals with autism spectrum disorder (ASD). Genes whose products regulate endogenous melatonin modify sleep patterns and have been implicated in ASD. Genetic factors likely contribute to comorbid expression of sleep disorders in ASD. We studied a clinically unique ASD subgroup, consisting solely of children with comorbid expression of sleep onset delay. We evaluated variation in two melatonin pathway genes, a*cetylserotonin O-methyltransferase* (*ASMT*) and *cytochrome P450 1A2* (*CYP1A2*). We observed higher frequencies than currently reported ($p < 0.04$) for variants evidenced to decrease *ASMT* expression and related to decreased CYP1A2 enzyme activity ($p \leq 0.0007$). We detected a relationship between genotypes in *ASMT* and *CYP1A2* ($r^2 = 0.63$). Our results indicate that expression of sleep onset delay relates to melatonin pathway genes.

Keywords Comorbidities · Genetic analyses · Phenotyping · Phenotypic subgroups · Biomarkers · Endophenotypes

Introduction

Autism spectrum disorder (ASD) is characterized by impairments in social communication and the presence of restricted and repetitive behavioral patterns (American Psychiatric Association 2013). Within this unified definition, the severity of clinical presentation is quite variable and many individuals express a number of comorbidities and endophenotypes (Gottesman and Gould 2003; Leyfer et al. 2006; Persico and Bourgeron 2006). It is generally accepted that underlying genetic factors play a role in susceptibility for ASD (Bailey et al. 1995; Cook Jr. 2001; Hallmayer et al. 2011). However, due to the diverse symptomatology among individuals with ASD, it is difficult to replicate genetic effects across datasets or identify clinically useful information related to outcomes from symptom treatment. It is possible that expression of various

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medical comorbidities in individuals with ASD can be explained by distinct underlying genetic effects (Alarcon et al. 2005, 2008; Bruining et al. 2010; Geschwind 2011; Hu et al. 2011; Peter et al. 2011; Whitehouse et al. 2011; Talebizadeh et al. 2013). By focusing on individuals with ASD also expressing a specific comorbidity, we may increase our ability to detect replicable genetic effects and identify clinically useful genetic information.

Expression of sleep disturbances provides a potential platform for identification of genetically similar subsets of ASD cases. Sleep disturbances are commonly observed in individuals with ASD, with prevalence estimates ranging from 50 to 80 % (Couturier et al. 2005; Krakowiak et al. 2008; Goldman et al. 2009; Souders et al. 2009). Current heritability estimates of sleep disruption indicate underlying genetic effects, with the proportion of variance in sleep problems related to heritable factors ranging from 20 to 64 % (Heath et al. 1990; Dauvilliers et al. 2005; Watson et al. 2006; Gehrman et al. 2011). Further, variation in genes whose products regulate endogenous melatonin modify sleep patterns in humans (Arendt 1998; Masana and Dubocovich 2001) and have been implicated in ASD (Cai et al. 2008; Melke et al. 2008; Hu et al. 2009; Jonsson et al. 2010; Braam et al. 2013).

Melatonin is produced via a series of enzymatic reactions that involves the acetylserotonin O-methyltransferase (ASMT) enzyme converting serotonin to melatonin (Ackermann and Stehle 2006). The involvement of the *ASMT* gene in relation to ASD etiology has been studied extensively (Toma et al. 2007; Cai et al. 2008; Melke et al. 2008; Jonsson et al. 2010; Wang et al. 2013). An ASD-risk haplotype was reported that includes two SNPs located in the promoter region, rs4446909 and rs5989681, and a third SNP, rs6644635, located in the 5′-untranslated region (UTR) of the only known functional isoform of *ASMT* (Melke et al. 2008; Botros et al. 2012). Further statistical studies have failed to replicate the associations of these common variants in *ASMT* with ASD risk (Toma et al. 2007; Wang et al. 2013). However, most of these previous studies do not report evidence of sleep disruption in any of the individuals screened, and the presence of sleep disturbance was not a primary focus for case criteria. Based on the reported high prevalence of sleep disturbances in ASD, it can be assumed that a proportion of individuals included in these ASD datasets exhibited sleep disturbances. It is therefore feasible that individuals who also expressed comorbid sleep disturbances contributed to the initial association with the *ASMT* risk haplotype, and the subsequent studies would have replicated the association were individuals categorized based on exhibition of comorbid sleep disturbances. By focusing solely on ASD cases expressing sleep disturbances, we may better understand the roles *ASMT* and melatonin regulation play in ASD etiology.

Once melatonin is produced, it is primarily metabolized by the liver enzyme, cytochrome P450 1A2 (CYP1A2) (Arendt et al. 1985; Arendt 1998). A potential relationship has been implicated between predicted slow-metabolizing alleles in *CYP1A2* and susceptibility to ASD with comorbid sleep problems (Braam et al. 2013). Melatonin supplementation is an emerging approach to treating ASD comorbid symptoms, including sleep initiation (Malow et al. 2012); however, some individuals are also non-responders or exhibit disappearing effectiveness (Braam et al. 2010; Rossignol and Frye 2011). Previous pharmacogenetic studies report that the altered efficacy and varied side effects of compounds used to treat neurological disease relate to individual genetic variation (Klepstad et al. 2005; Roses et al. 2007; Llerena et al. 2013). For example, numerous polymorphisms in the *CYP1A2* gene are reported to influence subsequent enzymatic activity (Sachse et al. 1999, 2003; Abnet et al. 2007; Lin et al. 2010; Kuo et al. 2013). By studying these polymorphisms in ASD cases with comorbid sleep disturbance we may identify factors useful for informing patient care.

Our aim was to understand the relationship between variation in melatonin pathway genes and expression of sleep problems in ASD. In this study, we tested the hypothesis that individuals with ASD and comorbid sleep onset delay would harbor a greater load of variation in genes related to maintenance of endogenous melatonin levels than has been previously reported in ASD.

Methods

Dataset Demographics

We screened DNA from a subset of children initially recruited for a larger melatonin trial. Details of the initial patient recruitment and melatonin trials were described previously (Malow et al. 2012). The entire evaluated genetic dataset included 15 unrelated children, ages 3–9 years old (Tanner Stage 1). Ascertainment for this study concluded in 2011, prior to the publication of the new DSM-V diagnostic criteria therefore a clinical diagnosis of ASD was based on a clinician interview that incorporated DSM-IV-TR criteria (American Psychiatric Association 2000). Diagnosis of ASD was confirmed via the Autism Diagnostic Observation Schedule (ADOS) (Lord et al. 1989) carried out by a trained psychologist. All children underwent a several week assessment period consisting of a comprehensive medical evaluation to address medical comorbidities and had a comprehensive sleep interview, followed by structured parent education (involving the primary caregiver, usually the mother) to provide instructions on daytime and evening habits to

Table 1 Dataset Demographics

Individuals	*CYP1A2* genotypes	*ASMT* sequences	Responsive to supplemental melatonin
Genetic sample demographics			
Male	12	12	9
Female	2	3	2
Total	14	15	11

Reported are gender breakdowns for the dataset included in genetic analyses of *CYP1A2* and/or *ASMT*. Inclusion criteria was as follows: clinician diagnosis of ASD, confirmed with ADOS, sleep onset delay $\geq$ 30 min on $\geq$ 3 nights/week, age between 3 and 9 years old, free of psychotropic medications, unrelated of European ancestry, no other diagnosed medical comorbidities. In addition, the subset of individuals who were also enrolled in a melatonin trial are indicated under the column 'Responsive to Supplemental Melatonin' as all children included in this trial were responsive

promote sleep (e.g., establishment of a bedtime routine, limiting electronic devices prior to bedtime) (Malow et al. 2013). The rationale for providing sleep education was to ensure that sleep difficulties did not simply reflect poor sleep hygiene. Children were confirmed to have sleep onset delay of at least 30 min at baseline on $\geq$3 nights per week, and none had sleep disturbance limited to specific seasons. There were twelve males and three females, and parent-reported race was 'White' (Table 1). Individuals were currently free of psychotropic medications. Two DNA samples were extracted from buccal swabs, the remaining 13 DNA samples were extracted from patient blood, using QIagen Puregene® chemistry on the Autopure® platform at the Vanderbilt DNA Resources Core.

Eleven of these 15 children were subsequently enrolled in the melatonin trial (Malow et al. 2012) and were initially started on liquid placebo for 2 weeks to obtain baseline data (Table 1). Individuals were then treated with 1 mg of supplemental liquid melatonin (Natrol®) for 3 weeks. If sleep latency, as measured by actigraphy, remained above 30 min on $\geq$2 nights in at least one of the treatment weeks, the dosage was increased to 3 mg for three more weeks. Sleep latency of all 11 children improved following treatment with supplemental melatonin at either 1 or 3 mg.

ASMT Mutation Screening

The *ASMT* gene is located on the pseudoautosomal region of chromosome Xp22.3/Yp11.3 and encodes an endoplasmic reticulum membrane protein expressed strongly in the pineal gland and retina. The enzyme catalyzes the transfer of a methyl group onto N-acetylserotonin, producing melatonin. We screened DNA from all 15 individuals for mutations in the exonic regions of *ASMT*. A region of *ASMT* including promoter B, the 5′-UTR, and exon 1B, was amplified via polymerase chain reactions (PCR) using the following primers: forward 5′-AAAAGGGGTCTCAC TATGTTGC-3′; reverse 5′-TGGAACGTGAGTGTGAT GAAC-3′. For all other regions, we used previously reported primers and PCR conditions to amplify the remaining exons and exon boundaries (Jonsson et al. 2010). Amplified products were purified from reactions with the QIAquick® PCR Purification Kit and Sanger sequenced at the Vanderbilt DNA Sequencing Facility and GenHunter® Corporation. Exon sequences were aligned in Sequencher® to the NCBI RefSeq gene sequence for isoform 1, NM_001171038. Alternatively spliced exons 6 and 7 were aligned to the NCBI RefSeq sequence for isoform 2, NM_004043. Base-pair changes from reference were detected in Sequencher, and presence of genotypes of interest at each SNP was verified by analyzing raw sequence chromatograms. The linkage disequilibrium (LD) map for SNPs of interest in this region was calculated using pairwise measures of the squared correlation coefficient (r^2) with Haploview (Barrett et al. 2005). Significance for genotype correlations were determined using pairwise correlation calculations in STATA 11.2 (College Station, TX, USA) (Statacorp 2009).

Genotype frequencies observed in the full dataset of 15, and in the subset of 11 children who were responsive to supplemental melatonin, were compared to current reports in individuals with ASD by calculating risk differences using Fisher's exact *p* value and the Woolf approximation to calculate standard errors and the 95 % confidence intervals (CI) around the odds ratio (OR) in STATA 11.2 (College Station, TX, USA) (Statacorp 2009).

CYP1A2 SNP Selection and Genotyping

The *CYP1A2* gene is located on chromosome 15q24.1 and encodes an endoplasmic reticulum membrane protein expressed strongly in the liver. The enzyme detoxifies high-energy aromatic proteins by adding oxygen groups and converting them to radical cations. A total of seven SNPs were considered in this analysis (Table 2). All SNPs had previous evidence for affecting CYP1A2 enzyme activity. Genotyping was performed as part of a Sequenom iPLEX® Goldpool according to the manufacturer's instructions. All samples were genotyped twice, with three quality control samples duplicated within and between plates, and genotypes were verified for concordance. One sample (of 15 total), had poor genotype efficiency and was not included in results reported for *CYP1A2*. No SNPs violated Hardy–Weinberg equilibrium in the evaluated samples. The LD map for SNPs of interest in this region was calculated as described above for *ASMT*. *CYP1A2* genotypes were compared to the *CYP1A2*1A* reference sequence (Thorn et al. 2012).

Table 2 Evaluated *CYP1A2* SNPs

SNP	Chromosomal location	Alleles	Function	Haplotype(s)	Predicted effect on metabolic activity
rs2069514	chr15: 75038220	G>A	Unknown	*1C, *1L	Decrease
rs12720461	chr15: 75041351	C>T	Intronic	*1 K	Decrease
rs762551	chr15: 75041917	C>A	Intronic	*1F, *1 J, *1 K, *1L, *1 M, *1 N, *1P, *1Q, *1R, *1 V, *1 W, *17, *21	Increase
rs2472304	chr15: 75044238	G>A	Intronic	*1 M, *1Q, *17	Decrease
rs72547516	chr15: 75044578	A>T; A>G	Missense	*4	Decrease
rs28399424	chr15: 75047169	C>T	Missense	*6	Decrease
rs2470890	chr15: 75047426	C>T	Synonymous	*1B, *1G, *1H, *1L, *1 N, *1P, *1R, *1S, *1T, *1U, *3, *8, *15, *16, *17, *18, *19, *20, *21	Decrease

Reported are the dbSNP ids, chromosomal locations based on the current genomic build (GRCh37.p10), base-pair changes compared to the *CYP1A2*1A* reference sequence, functional predictions based on SNP location, associated haplotypes, and previously reported effects on CYP1A2 enzyme activity for each SNP genotyped in our patients. Haplotypes are those reported by PharmGKB

Genotype frequencies observed in the full dataset of 14 and in the subset of 10 children who were responsive to supplemental melatonin were compared to current reports for populations of similar genetic ancestry (i.e. European) as described above for *ASMT*.

Results

ASMT Mutation Screening

We observed seven previously reported SNPs located within exon boundaries for *ASMT* that were polymorphic in our individuals (Table S1). All individuals were either homozygous or heterozygous for dysfunctional variants in the previously predicted ASD-'risk' haplotype (i.e. rs4446909, rs5989681, rs6644635) (Melke et al. 2008). For these SNPs, we compared genotype and allele frequencies in our individuals to those currently reported for individuals with ASD where sleep onset delay was not indicated (Toma et al. 2007; Melke et al. 2008; Jonsson et al. 2010; Wang et al. 2013). We observed similar and, in some cases, higher allele and genotype frequencies for the dysfunctional *ASMT* polymorphisms compared to current estimates in ASD (Table 3; Table S1; Table S2). In the entire genetic dataset, we observed a higher frequency of individuals with genotypes representative of the 'risk' haplotype than currently reported (Table 3). LD calculations indicated genotypes for the promoter B polymorphisms were strongly correlated ($r^2 = 0.84$, $p < 0.0001$) in the entire evaluated dataset (Fig. 1a). However, we observed minimal correlations across all three SNPs in the previously reported 'risk' haplotype ($r^2 = 0.18$–0.21) in these samples. In the smaller subset of children who were all responsive to supplemental melatonin, LD calculations indicated genotypes for the promoter B polymorphisms were perfectly correlated ($r^2 = 1.00$, $p < 0.0001$) (Fig. 1b). We did not observe any novel or previously reported rare point mutations in our individuals (Jonsson et al. 2010; Wang et al. 2013).

CYP1A2 SNP Genotyping

Six of the seven SNPs that were genotyped in *CYP1A2* were polymorphic, compared to *CYP1A2*1A*, in the 14 evaluated individuals. We observed substantially higher rates of polymorphism at these SNPs than in the one previous report for individuals with ASD (Braam et al. 2013). As very little is currently reported regarding *CYP1A2* variation in individuals with ASD, and to ensure there was not a relationship between *CYP1A2* haplotypes and European ancestry, we compared our estimates to those reported for evaluated populations of European ancestry (http://www.ncbi.nlm.nih.gov/snp/). We observed significantly higher frequencies for three variant alleles related to decreased CYP1A2 enzyme activity at SNP rs2069514 (aka *CYP1A2*1C*), SNP rs72547516 (aka *CYP1A2*4*), and SNP rs28399424 (aka *CYP1A2*6*) (Tables 2, 4; Table S3). The remaining SNPs were observed at similar frequencies.

In the entire evaluated dataset, LD calculations indicated a modest correlation between genotypes at rs762551 and rs2470890 ($r^2 = 0.49$, $p = 0.0002$) and a stronger correlation between genotypes at rs2472304 and rs2470890 ($r^2 = 0.64$, $p < 0.0001$). There was also a modest correlation between rs762551 and rs2472304 ($r^2 = 0.31$, $p = 0.0031$) (Fig. 1a). In the subset of individuals responsive to melatonin, LD calculations still suggest a modest correlation between genotypes at rs762551 and rs2470890 ($r^2 = 0.40$, $p = 0.0047$) and perfect correlation between genotypes at rs2472304 and rs2470890 ($r^2 = 1.00$, $p < 0.0001$). The correlation between rs762551 and rs2472304 was also stronger in this analysis subset ($r^2 = 0.40$, $p = 0.0047$) (Fig. 1b). LD calculations

Table 3 Dysfunctional *ASMT* SNPs in ASD with sleep onset delay (SOD)

Promoter B/5′UTR	ASD with SOD	ASD Melke et al. 2008	ASD Toma et al. 2007			ASD Wang et al. 2013
	US Caucasian n = 15	European Caucasian n = 278	Finnish n = 127	Italian n = 69	UK n = 194	Han Chinese n = 398
SNP (Allele)						
rs4446909 (G)	0.73	0.77	0.89	0.76	0.76	0.70
rs5989681 (G)	0.70	0.73	0.87	0.72	0.72	0.57
rs6644635 (C)	0.67	0.65	0.64	0.61	0.62	0.77
Haplotypes						
GGC	0.53	0.36	0.51	0.32	0.32	0.34
p value		0.04*	0.49	0.02*	0.02*	0.03*
OR (95 % CI)		2.03 (0.97, 4.25)	1.09 (0.51, 2.33)	2.44 (1.10, 5.44)	2.43 (1.15, 5.14)	2.21 (1.06, 4.60)
GGT	0.17	0.36	0.35	0.39	0.38	0.22
p value		0.02*	0.03*	0.01*	0.01*	0.33
OR (95 % CI)		0.36 (0.13, 0.94)	0.37 (0.14, 1.00)	0.31 (0.11, 0.87)	0.33 (0.12, 0.88)	0.71 (0.27, 1.88)
ACC	0.10	0.21	0.11	0.22	0.22	0.28
p value		0.10	0.58	0.11	0.09	0.02*
OR (95 % CI)		0.42 (0.12, 1.40)	0.90 (0.26, 3.15)	0.40 (0.11, 1.41)	0.40 (0.12, 1.34)	0.29 (0.09, 0.95)
GCC	0.00	0.06	0.02	0.05	0.06	0.14
p value		0.17	0.57	0.24	0.17	0.01*
OR (95 % CI)		–	–	–	–	–
ACT	0.17	NR	NR	NR	NR	NR

Reported are frequencies for alleles and predicted haplotypes at the promoter B and 5′-UTR SNPs in *ASMT*. No allele frequencies were significantly different ($p < 0.05$) in our dataset compared to other reports in ASD. Observed haplotype frequencies were significantly different; asterisks denote a significant difference ($p \leq 0.04$). Reported Fisher's exact *p* values are uncorrected. OR indicates the Woolf approximation of the odds ratio calculating the odds of having the noted haplotype given the individual with ASD has comorbid sleep onset delay (i.e. our evaluated dataset). We observed no patients with the GCC haplotype, therefore, no odds ratios could be calculated. NR indicates this haplotype was not reported in the studies we used for comparison. The GGC haplotype is more frequent in our dataset compared to current estimates in ASD, with the exception of the previously evaluated Finnish ASD population. Presence of alleles representative of this haplotype have been shown to relate to decreased levels of *ASMT* transcript production in individuals with ASD, relative to other haplotypes. Numbers (n) represent the sample size of the indicated dataset

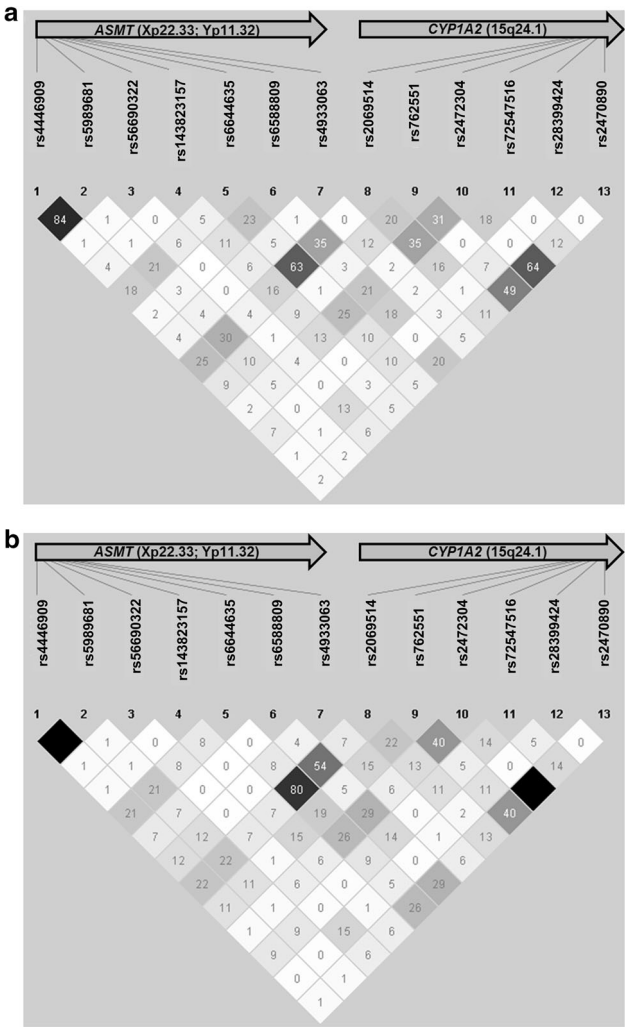

Fig. 1 Haplotype block structure of *ASMT* and *CYP1A2*. Reported are pairwise LD calculations (r^2) between all SNPs in *ASMT* and *CYP1A2* that were polymorphic in our dataset. *Darker shading* indicates stronger correlations. When no value is reported, $r^2 = 1.0$. *Arrows* indicate the gene names, with corresponding chromosomal locations, in which SNPs are located and the 5′ to 3′ direction of each transcript. **a** LD plot in the entire evaluated dataset comprised of individuals with ASD and comorbid sleep onset delay, and **b** in a subset of these individuals who were responsive to supplemental melatonin. A potential relationship was observed between SNP rs6644635 in *ASMT* and SNP rs2069514 in *CYP1A2* in the entire evaluated dataset. Upon evaluation of only those individuals who were also included in our melatonin trial (all were responsive) the relationship between SNPs rs6644635 and rs2069514 in *CYP1A2* became stronger. Further, a relationship was also observed between SNP rs6644635 and rs6588809 in this subset of our larger dataset

suggested that genotypes for the remaining SNPs we evaluated were independent in these individuals.

Comparison of ASMT and CYP1A2 Genotypes

In the entire evaluated dataset, we observed a strong correlation between genotypes at SNP rs6644635, located in the 5′-UTR of the functional *ASMT* isoform, and SNP rs2069514, located in a transcription factor binding site within the promoter element for *CYP1A2* ($r^2 = 0.63$, $p = 0.0408$) (Fig. 1a). This relationship between *ASMT* and *CYP1A2* increased ($r^2 = 0.80$, $p = 0.0199$) when only assessing children who were also included in our melatonin trial and who were responsive to treatment (Fig. 1b).

Discussion

In this work, we found that children with ASD and comorbid sleep onset delay harbored a greater load of dysfunctional variation in genes related to the melatonin pathway, especially with regard to *CYP1A2*, as compared to those previously reported in the literature. We also observed a correlation between genotypes in *ASMT* and in *CYP1A2* in these samples. When evaluating only the subset of children who responded to treatment with supplemental melatonin, this connection between genotypes in *ASMT* and *CYP1A2* was even stronger. These results further implicate a relationship between the *ASMT* and *CYP1A2* genes and expression of comorbid sleep onset delay that improves with melatonin treatment in ASD.

Expression of Sleep Disturbances in ASD is Genetically Relevant Information

Our evaluated dataset was unique in that the children with ASD were also diagnosed with sleep onset delay. Their diagnosis of ASD was carefully established. All underwent a comprehensive clinical evaluation to address medical, neurological and psychiatric comorbidities and all of their parents received sleep education designed to promote good sleep habits in the children. These measures helped ensure that their sleep onset delay was not simply due to a medication, medical, neurological or psychiatric condition, or poor sleep habits. Further, a subset of 11 children from the 15 included in the entire genetic dataset were participating in a clinical trial of supplemental melatonin. All 11 children responded to supplemental melatonin treatment at relatively low doses (1 or 3 mg) and were otherwise medication-free. We also evaluated this subset of children, 9 males and 2 females, who were responsive to melatonin treatment separately from the original genetic sample of 15 children for whom we did not have treatment data available (Table 1, Table S3, Table S4, Fig. 1b). Our results indicated that sleep disorder data are useful for genetic studies of the melatonin pathway in ASD. If *ASMT* and *CYP1A2* play important roles in expression of sleep disturbance in ASD we would expect that our sample consisting solely of children with sleep onset delay would harbor a greater load of dysfunctional variation compared to datasets where no sleep-related information was

Table 4 Observed genotypes for evaluated *CYP1A2* SNPs in ASD with sleep onset delay (SOD)

CYP1A2 SNP	Genotype frequencies ASD with $SOD_{Current\ Dataset}$ n = 14			Allele frequencies ASD with $SOD_{Current\ Dataset}$ n = 14		Allele frequencies European ancestry n = variable	
rs2069514	**GG**	**GA**	**AA**	**G**	**A**	**G**	**A**
	0.21	0.79	0.00	0.607	0.393	0.919	0.081
p value (dbSNP population)				*0.0007** ($n_{SNP500CANCER} = 31$)			
OR (95 % CI)				*7.38 (2.25, 24.19)*			
rs12720461	**CC**	**CT**	**TT**	**C**	**T**	**C**	**T**
	1.00	0.00	0.00	1.000	0.000	1.000	0.000
p value (dbSNP population)				No difference ($n_{HapMap\text{-}CEU} = 60$)			
OR (95 % CI)							
rs762551	**CC**	**CA**	**AA**	**C**	**A**	**C**	**A**
	0.07	0.36	0.57	0.250	0.750	0.279	0.721
p value (dbSNP population)				0.4567 ($n_{HapMap\text{-}CEU} = 226$)			
OR (95 % CI)				0.85 (0.35, 2.06)			
rs2472304	**GG**	**GA**	**AA**	**G**	**A**	**G**	**A**
	0.07	0.79	0.14	0.464	0.535	0.336	0.664
p value (dbSNP population)				0.1304 ($n_{HapMap\text{-}CEU} = 226$)			
OR (95 % CI)				0.60 (0.28, 1.28)			
rs72547516	**AA**	**AT**	**TT**	**A**	**T**	**A**	**T**
	0.71	0.29	0.00	0.857	0.143	0.998	0.002
p value (dbSNP population)				*<0.00001* ($n_{ClinSeq} = 662$)*			
OR (95 % CI)				*173.39 (36.81, 816.82)*			
rs28399424	**CC**	**CT**	**TT**	**C**	**T**	**C**	**T**
	0.14	0.14	0.14	0.786	0.214	0.999	0.001
p value (dbSNP population)				*<0.00001* ($n_{ClinSeq} = 662$)*			
OR (95 % CI)				*360.82 (41.67, 3124.08)*			
rs2470890	**CC**	**CT**	**TT**	**C**	**T**	**C**	**T**
	0.14	0.57	0.29	0.428	0.571	0.376	0.624
p value (dbSNP population)				0.3521 ($n_{ClinSeq} = 651$)			
OR (95 % CI)				0.80 (0.38, 1.71)			

Reported are frequencies for genotypes and alleles at SNPs genotyped in *CYP1A2*. Asterisks indicate SNPs where variant allele frequencies were significantly increased ($p \leq 0.0007$) compared to populations of European ancestry reported in dbSNP. Reported Fisher's exact p-values are uncorrected. O.R. indicates the Woolf approximation of the odds ratio calculating the odds of having the noted allele given the individual has ASD and comorbid sleep onset delay (i.e. current dataset). Numbers (n) represent the number of individuals for which frequencies are reported in each dataset. In the case where allele frequency data were available in dbSNP for more than one population of European ancestry, we report our results compared to the largest dataset evaluated. We observed significantly higher frequencies for alleles related to decreased enzymatic activity for the three SNPs indicated in bold italics

evaluated. This was especially true for polymorphisms in *CYP1A2*.

Sleep Onset Delay in ASD is Potentially Related to Reduced ASMT Gene Expression

Interestingly, we observed that all of our individuals with ASD and sleep onset delay were either homozygous or heterozygous for dysfunctional variants in SNPs predicted to alter *ASMT* transcript production. This indicates that sleep onset delay in ASD is related to lower levels of *ASMT* gene expression. Decreases in *ASMT* expression have been attributed to homozygous presence of 'risk' alleles at the promoter B SNPs, rs4446909 and rs5989681 (Melke et al. 2008). We have evidence from in vitro studies performed in cell lines from ASD individuals suggesting decreased *ASMT* expression is attributable to variant genotypes (whether homozygous or heterozygous) at the promoter B SNPs and at the 5′-UTR SNP, rs6644635 (Veatch and Haines 2013). Another study in individuals with depression also reported effects on *ASMT* expression attributed to variation at the promoter B SNPs (Galecki et al. 2010). While decreased expression related to heterozygous and homozygous rs4446909 ASD-'risk' genotypes was observed, an inverse relationship between heterozygous and homozygous rs5989681 ASD-'risk' genotypes and

ASMT expression was also reported. It is notable the variable effect of genotypes at this SNP was observed in individuals with recurrent depressive disorder, not ASD. It is possible the effect of these SNPs on *ASMT* expression varies under different genetic backgrounds. This suggests there may still be undiscovered epigenetic regulation and gene–gene interactions affecting expression of *ASMT*.

A Connection Between Slow-Metabolizing Alleles in CYP1A2 and Sleep Onset Delay in ASD

We observed substantially higher rates of alleles related to decreased CYP1A2 activity compared to populations of European ancestry in dbSNP. In the only previously published study evaluating *CYP1A2* polymorphisms in seven ASD individuals clinically diagnosed as slow melatonin metabolizers, the authors genotyped six SNPs tagging the *CYP1A2* variants we also evaluated (Braam et al. 2013). Of the seven individuals with ASD and comorbid sleep disturbance genotyped in the Braam et al. study, only four were polymorphic at one out of six evaluated SNPs; three were observed polymorphic at *CYP1A2*1F* (aka rs762551), and one was observed polymorphic at *CYP1A2*1C* (aka rs2069514). The individuals included in this previous study all exhibited disappearing effectiveness of melatonin treatment and the authors report this phenomenon was related to slow melatonin metabolism. The authors suggested a mechanism in that increasing levels of melatonin in the system results in loss of circadian rhythms and eventual loss of supplemental melatonin effectiveness. Despite our observations of higher frequencies for SNPs related to decreased enzymatic activity, we currently have no evidence indicating these children are slow melatonin metabolizers ($T^{1/2} > 2$ h) (Goldman et al. 2014). We also did not observe a relationship between the number of 'slow metabolizing' alleles observed in *CYP1A2* and either the severity of sleep onset delay, or the effective melatonin dosage in our dataset.

We also observed that 57 % of the individuals evaluated were homozygous AA at rs762551. The *CYP1A2*1F* haplotype, tagged by rs762551, has been associated in many studies with altered phenotypes, and homozygous presence of the A allele (i.e., the reference allele) at this SNP has been associated with increased *CYP1A2*-related metabolism and reports of adverse events following antipsychotic use (Ozdemir et al. 2001; Eap et al. 2004; Ghotbi et al. 2007; Laika et al. 2010). Six individuals were heterozygous at this SNP. We did not observe any individuals who were homozygous CC at this SNP (e.g., the genotype not related to increased enzymatic activity). Although the AA genotype at this *CYP1A2* variant is generally thought to relate to increased metabolism, when other variants are considered, specifically those in the *CYP1A2*K* haplotype which also includes rs12720461, decreased metabolic activity is observed (Aklillu et al. 2003). In the above mentioned Braam et al. (2013) study evaluating *CYP1A2* polymorphisms in individuals clinically diagnosed as slow melatonin metabolizers, six individuals were observed with the AA genotype at rs762551, but none were polymorphic at SNP rs12720461. None of our individuals were polymorphic at rs12720461. It is important to note that the fast metabolism phenotype related to the rs762551AA genotype has only been observed in adults under induction by smoking or habitual coffee intake (Djordjevic et al. 2008; Thorn et al. 2012). It is possible that there is a gene-environment interaction between the rs762551AA genotype (i.e. the *CYP1A2*F* haplotype) and caffeine consumption and that there are no genotype-specific effects on CYP1A2 enzymatic activity without this environmental influence. This is very interesting given that caffeine intake has been shown to influence sleep patterns (Irish et al. 2013; Lodato et al. 2013; Lohsoonthorn et al. 2013) and that minimizing caffeine consumption at specific times is an essential part of sleep education (Malow et al. 2013).

Study Limitations

This study has several limitations. First, as a result of clinical ascertainment procedures (individuals were ascertained based on presence of a sleep disturbance), we did not have a well-defined control group for comparison. The ideal control group would contain individuals of European ancestry having ASD with no evidence of sleep onset delay. As such, we compared our results for *ASMT* to previous reports in ASD and for *CYP1A2* to datasets available in dbSNP. It is notable we have no relevant sleep or ASD data in the publicly available dbSNP datasets. However, as we compared our frequencies to the largest evaluated datasets available, the observation of higher rates for dysfunctional alleles at three *CYP1A2* SNPs in our small dataset suggests this gene is important for expression of sleep disturbance in some children with ASD. It is possible that our evaluated case dataset represents a biased genetic sample; however, if there is no relationship between dysfunctional alleles in *CYP1A2* and sleep disturbance in ASD, higher frequencies for these alleles should have been observed in the much larger dbSNP datasets compared to our small sample.

In addition, the subset of 11 individuals who participated in the melatonin trial were all responsive to treatment. As a result, we were unable to assess potential differences in *ASMT* and *CYP1A2* between responders and non-responders in this study. This is an important question we plan to evaluate further in future analyses.

While there is potential for medication use and environmental factors to influence sleep onset delay, we expect that by only including individuals having no other medical comorbidities and who were not using psychotropic

medications we have reduced the potential for these factors to affect sleep latency. Also, we expect to have minimized the environmental effect of poor sleep habits through parent sleep education. While the sample size evaluated in our study represents a very small cohort, the extensive phenotypic homogeneity and medical evaluations we established indicate that our findings may be relevant to similar children with ASD. Further, the necessary exclusion of *CYP1A2* genotypes for one sample due to poor genotyping efficiency may slightly limit the findings of higher frequencies for dysfunctional alleles in this gene and the relationship between genotypes in *ASMT* and *CYP1A2*. Of course, it will be necessary to try to replicate all of these results in a larger sample having relevant medical information available.

Our results support a relationship between melatonin pathway genes and expression of comorbid sleep disturbance in ASD. We expect that *ASMT* and *CYP1A2* play important roles in expression of sleep disturbance in ASD given that in general our dataset, consisting solely of children with sleep onset delay, harbored a greater load of dysfunctional variation compared to datasets where no sleep-related information was evaluated. The LD structure across SNPs in *ASMT* and *CYP1A2* in our subset of individuals predicts a potential gene–gene interaction. We observed a strong correlation between the dysfunctional 5′-UTR SNP, rs6644635, in *ASMT* and the dysfunctional promoter SNP, rs2069514, in *CYP1A2*. Our findings suggest a mechanism connecting lower levels of *ASMT* transcript production with reduced CYP1A2 metabolic activity in children with ASD and comorbid sleep onset delay. To fully understand this underlying relationship to ASD etiology, it will be necessary to evaluate larger ASD datasets, focusing on children with comorbid sleep onset delay and melatonin pharmacokinetic data.

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