Published in final edited form as:

J Inherit Metab Dis. 2014 May ; 37(3): 415–420. doi:10.1007/s10545-013-9672-5.

Decreased Cerebral Spinal Fluid Neurotransmitter Levels in Smith-Lemli-Opitz Syndrome

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Summary

Smith-Lemli-Opitz Syndrome (SLOS) is an autosomal recessive, multiple congenital anomaly syndrome with cognitive impairment and a distinct behavioral phenotype that includes autistic features. SLOS is caused by a defect in β -hydroxysterol ⁷-reductase which leads to decreased cholesterol levels and elevated cholesterol precursors, specifically 7- and 8-dehydrocholesterol. However, the pathological processes contributing to the neurological abnormalities in SLOS have not been defined. In view of prior data suggesting defects in SLOS in vesicular release and given the association of altered serotonin metabolism with autism, we were interested in measuring neurotransmitter metabolite levels in SLOS to assess their potential to be used as biomarkers in therapeutic trials. We measured cerebral spinal fluid levels of serotonin and dopamine metabolites, 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) respectively, in 21 SLOS subjects. Results were correlated with the SLOS anatomical severity score, Aberrant Behavior Checklist scores and concurrent sterol biochemistry. Cerebral spinal fluid (CSF) levels of both 5HIAA and HVA were significantly reduced in SLOS subjects. In individual patients, the levels of

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Conflicts of Interest: Susan Sparks, Chris Wassif, Halima Goodwin, Sandra Conley, Diane Lanham, Andrea Gropman, Elaine Tierney and Forbes Porter report no conflicts of interest.

Lisa Kratz is employed in the Biochemical Genetics Laboratory, Kennedy Krieger Institute, Baltimore, MD. This laboratory performs clinical testing for sterols.

Keith Hyland is employed by Medical Neurogenetics, Atlanta, GA. Medical Neurogenetics performs commercial clinical testing of CSF neurotransmitter metabolites.

Author Contributions: SES, CAW, AG, ET and FDP were involved in the design, approval and implementation of the clinical protocol. SES, HG, SKC, ET and FDP were involved with protocol coordination, patient care and collection of both patient data and biomaterials. CAW, LEK and KH provided expert input on and were involved in analyte analysis. SES, CAW, DCL, AG, ET and FDP were involved in the analysis of the data. SES, CAW and FDP were involved in the initial drafting of the manuscript. All authors read and provided input on the manuscript.

both 5HIAA and HVA were reduced to a similar degree. CSF neurotransmitter metabolite levels did not correlate with either CSF sterols or behavioral measures. This is the first study demonstrating decreased levels of CSF neurotransmitter metabolites in SLOS. We propose that decreased levels of neurotransmitters in SLOS are caused by a sterol-related defect in synaptic vesicle formation and that CSF 5HIAA and HVA will be useful biomarkers in development of future therapeutic trials.

Introduction

Smith-Lemli-Opitz Syndrome (SLOS, OMIM #270400) is an autosomal recessive, cognitive impairment, multiple congenital anomaly syndrome originally described by Smith, Lemli, and Opitz in 1964 (Smith, Lemli et al. 1964). The incidence of SLOS is approximately 1:40,000 live births (Porter 2000). Children with SLOS have typical facial features (ptosis, broad nasal bridge, short upturned nose, microretrognathia), microcephaly, failure to thrive, developmental delays, intellectual disability, syndactyly of the second and third toes, and multiple congenital anomalies including central nervous system, cardiac, pulmonary, gastrointestinal, renal and genitourinary malformations (reviewed in [2]). The biochemical abnormality, increased plasma and tissue levels of 7- and 8-dehydrocholesterol and variably low cholesterol level, was reported in 1993 (Irons, Elias et al. 1993), followed by identification of the molecular defect in the gene encoding 3 β -hydroxysterol 7-reductase (*DHCR7*) in 1998 (Fitzky, Witsch-Baumgartner et al. 1998; Wassif, Maslen et al. 1998; Waterham and Wanders 2000). Although both cholesterol deficiency and toxic effects of accumulating precursors likely contribute to SLOS pathology, the extent to which each of these biochemical abnormalities contribute to the developmental malformations and cognitive problems have not been fully delineated.

The cognitive deficits in SLOS range from profound mental retardation to borderline normal intelligence (Mueller, Patel et al. 2003), and may be secondary to developmental malformations or functional deficits due to altered sterol biochemistry in the central nervous system. Congenital anomalies of the central nervous system include microcephaly in most individuals, as well as defects of myelination, malformations of the corpus callosum, abnormalities of the cerebellum including Dandy-Walker variant, and at the severe end of the spectrum, holoprosencephaly (Ryan, Bartlett et al. 1998; Kelley and Hennekam 2000; Caruso, Poussaint et al. 2004; Lee, Conley et al. 2013; Lee, Yoshida et al. 2013). Hypotonia is extremely common in infants and young children with SLOS, whereas older children often exhibit hypertonia.

In this study, we evaluated 21 patients with SLOS ranging in age from 4 to 17 years. Subjects were characterized clinically, molecularly, and biochemically. Because secondary abnormalities in CSF serotonin and catecholamine levels have been identified in a number of neurological disorders (Hyland 2008), we measured CSF levels of 5-hydroxyindoleacetic acid (5HIAA, serotonin metabolite) and homovanillic acid (HVA, dopamine metabolite) and found that both were decreased in SLOS. These data may give insight into the neurological and behavioral aspects of SLOS.

Patients and Methods

Patients, ethical approval and phenotyping

The 21 SLOS subjects in this study were enrolled in a placebo-controlled, cross-over trial of simvastatin therapy (NCT00064792). Data in this paper represent the base-line evaluation. All patients were evaluated at both the National Institutes of Health Clinical Center and Kennedy Krieger Institute in Baltimore. This study was approved by the Eunice Kennedy Shriver National Institute of Child Health and Human Development and the Kennedy Krieger Institute Institutional Review Boards, and written informed consent was obtained from either a parent or guardian. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. The clinical diagnosis of SLOS was confirmed by biochemical and molecular testing. Anatomical severity scores based on malformations were calculated as previously described (Kelley and Hennekam 2000). Behavioral abnormalities were ascertained using the Aberrant Behavior Checklist, a rating scale that can be used to ascertain for therapeutic effects in individuals with intellectual impairment (Aman, Singh et al. 1985).

Biochemical and Molecular Analysis

Molecular analysis was performed as previously described (Krakowiak, Nwokoro et al. 2000) or by GeneDx (Gaithersburg, MD). Sterol levels were determined by gas chromatography/mass spectrometry as previously described (Kelley 1995). Due to the rostral-caudal gradient of the CSF neurotransmitters, the first 0.5 ml of CSF collected was used to measure the biogenic amines. CSF monoamine metabolites, 5HIAA and HVA were measured using isocratic reverse-phase liquid chromatography with in-series dual cell colorimetric electrochemical and fluorescence detection (Hyland, Surtees et al. 1993).

Results

Subject cohort

Twenty-one SLOS subjects undergoing initial evaluation for a simvastatin therapeutic trial were included in this study. Patient demographics, severity score, and *DHCR7* genotype are presented in Table 1. Patients ranged in age from 4 to 17 years, and 13 (62%) were male. All subjects in this study had mild to classical SLOS with severity scores ranging from 6 to 28, out of a maximum score of 100. Severely affected cases were not studied, because they did not meet safety inclusion criteria for the simvastatin trial. All patients were on dietary cholesterol supplementation (150 mg/kg/d of crystalline cholesterol in OraPlus). Nine of the patients were heterozygous for the common splice acceptor c.964-1G>C mutation. This is consistent with this mutation accounting for approximately a third of identified mutations in SLOS patients. Plasma cholesterol and dehydrocholesterol levels ranged from 47 to 162 mg/dL and 1.8 to 289 mcg/mL, respectively. CSF cholesterol and dehydrocholesterol levels ranged from 0.96-3.66 and 0.011-0.232 mcg/ml, respectively. For comparison mean CSF cholesterol level in eight pediatric samples was 2.73 ± 1.11 mcg/ml and dehydrocholesterol levels were 0.001 mcg/ml.

CSF neurotransmitters

We measured CSF 5HIAA and HVA levels, metabolites of serotonin and dopamine, respectively, in this SLOS cohort. All 21 patients had 5HIAA and HVA levels lower than the age and gender appropriate mean, and ten (48%) subjects had levels more than two standard deviations below the mean (Figure 1A). Z-scores for 5HIAA levels ranged from -0.1 to -3.9, and for HVA from -0.9 to -3.5. Both neurotransmitter metabolites decreased proportionately to each other (Figure 1B, $r^2=0.74$, $p<0.0001$), suggesting a common mechanism underlying the decrease in these two metabolites. Because tetrahydrobiopterin is a cofactor for the synthesis of both dopamine and serotonin, CSF tetrahydrobiopterin levels were measured in 15 of the 21 subjects. The mean CSF tetrahydrobiopterin level was 18.4 nmol/L, and no level was less than the lower limit of normal of 9 nmol/L. Neither 5HIAA nor HVA levels correlated with either cholesterol (Figures 1C and 1D) or DHC levels (Figure 1E and 1F). Similarly, neither 5HIAA nor HVA showed significant correlations individually with 7-dehydrocholesterol ($p=0.18$ and $p=0.96$, respectively), 8-dehydrocholesterol ($p=0.46$ and $p=0.72$, respectively), or the DHC/total sterol ratio ($p=0.20$ and 0.81 , respectively).

We explored whether or not the decreased neurotransmitter levels correlated with the phenotypic characteristics of SLOS. Neither absolute levels nor z-scores for 5HIAA ($p=0.50$ and $p=0.35$, respectively) or HVA ($p=0.94$ and 0.83 , respectively) showed significant correlation with the SLOS severity score. Although absolute occipital frontal circumference (OFC) measurements did not correlate 5HIAA or HVA levels, HVA z-scores had a positive correlation ($p<0.05$, $r^2=0.26$) with OFC z-scores, whereas 5HIAA showed no correlation ($p=0.25$) with OFC z-scores. We also evaluated behavioral aspects of SLOS, but found no significant correlations with any of the five subscales (Irritability and Agitation, Lethargy and Social Withdrawal, Stereotypic Behavior, Hyperactivity and Noncompliance, and Inappropriate Speech) of the Aberrant Behavior Checklist.

Discussion

In this study we found that CSF levels of 5HIAA and HVA were significantly lower compared to controls in subjects with SLOS, suggesting an abnormality in the synthesis, release, or metabolism of serotonin and dopamine. Interestingly, both neurotransmitter metabolites were proportionately decreased in individual subjects. This suggests a generalized defect of neurotransmitter synthesis, release or catabolism, rather than specific defects related to either serotonin or dopamine metabolism. Because microcephaly is present in most individuals with SLOS, it is possible that the decreased CSF HVA and 5-HIAA levels simply reflect the smaller brain size in more severely affected subjects. Using OFC as a proxy for brain size (Bartholomeusz, Courchesne et al. 2002), we found a positive correlation between CSF HVA levels and OFC; however, this was not observed for 5HIAA. Tetrahydrobiopterin and neopterin levels were normal, thus suggesting that there was not an overall decrease in amine related metabolites. It is unlikely that decreased synthesis or increased catabolism explain the high correlation between CSF 5HIAA and HVA levels in an individual patient. Serotonin and dopamine synthesis involves distinct biosynthetic pathways with serotonin being derived from tryptophan and dopamine being derived from

tyrosine. However, because tetrahydrobiopterin is a common cofactor for both tryptophan and tyrosine hydroxylase, we measured CSF tetrahydrobiopterin levels but found all values to be within the expected reference range. Although monoamine oxidase is involved in the catabolism of both serotonin and dopamine to 5HIAA and HVA, respectively, most serotonin is recycled by presynaptic neurons, and catabolism of dopamine to HVA involves the sequential action of both monoamine oxidase and catechol-O-methyltransferase. Given the differences in the catabolic pathways, it is unlikely that decreased monoamine oxidase activity accounts for the observed low levels of both 5HIAA and HVA. Folate deficiency is associated with decreased CSF levels of both 5HIAA and HVA, and Suretees et al (Suretees, Heales et al. 1994) demonstrated that a deficiency of 5-methyltetrahydrofolate in CSF was associated with decreased levels of 5HIAA and HVA. Unfortunately, in this current study, insufficient CSF was available for measurement of 5-methyltetrahydrofolate levels to determine if an abnormality of folate metabolism could underlie our observation of decreased 5HIAA and HVA levels in SLOS subjects. This is a hypothesis that can be tested in future studies. Both serotonin and dopamine are stored in presynaptic secretory granules and upon stimulation, are released into the synaptic cleft by exocytosis. Cholesterol is an important lipid component of synapses and plays a major role in synaptic vesicle release (Rosa and Fratangeli 2010). Dense granules are similar to synaptic vesicles in membrane composition and function. We have previously demonstrated a significant decrease in the number of synaptic vesicle-like dense granules in the pancreas, pituitary, and adrenal glands from *Dhcr7* mutant mice (Gondre-Lewis, Petrache et al. 2006). Furthermore, dense granules in the exocrine pancreas demonstrated abnormal morphology and impaired secretory regulation. It is thus possible that a defect in synaptic vesicle formation or exocytosis in neurons could contribute to the observation of decreased serotonin and dopamine metabolites in SLOS subjects. Analysis of synaptic vesicle number and morphology in *Dhcr7* deficient mice could provide a means to test this idea.

The findings reported in this study could be relevant to a number of prior studies suggesting that neurotransmitter function is impaired in SLOS. For example, the NMDA receptor response to glutamate in neurons from SLOS mutant mice is impaired (Wassif, Zhu et al. 2001), 7DHC may impair serotonin_{1A} receptor ligand binding (Singh, Paila et al. 2007), and there is an increase in the area and intensity of 5-HT immunoreactivity in the hindbrains of embryonic SLOS mice compared to matched wild type mice (Waage-Baudet, Lauder et al. 2003). The data reported in this paper supports the hypothesis that impaired neurotransmitter metabolism contributes to the SLOS phenotype.

One purpose of our study was to determine if we could correlate neurotransmitter metabolite levels with specific aspects of the SLOS behavioral phenotype. There is evidence that the serotonergic system is developmentally dysregulated in autism [reviewed in (Scott and Deneris 2005)], and possible dysregulation of this system in SLOS was particularly interesting given the presence of autistic symptoms in SLOS (Tierney, Nwokoro et al. 2001). A number of studies have measured 5-HIAA levels in autistic patients, although most of these studies have not shown differences between autistic patients and controls (reviewed in (Narayan, Srinath et al. 1993)). Thus, CSF 5-HIAA levels do not appear to be a good surrogate marker for autistic symptoms, and consistent with this, we were unable to correlate

5-HIAA levels with autistic symptoms in our SLOS subjects. We also found no correlation of CSF 5HIAA or HVA levels with behavioral abnormalities ascertained by the Aberrant Behavioral Checklist. However, the small size of this study, and, consequently, the low statistical power, is a major limitation in concluding definitively that there is a lack of correlation.

Although CSF 5HIAA levels in autism do not seem to be altered, there remains strong evidence that the serotonergic system is dysregulated in autism. Various studies of autistic individuals have found increased levels of serotonin (5-HT) both in the brain and systemically, altered 5-HT synthesis, and genetic polymorphisms in the 5-HT transporter (reviewed in (Scott and Deneris 2005)). In addition, some effective therapies for autism involve medications that modulate the serotonergic system. Cholesterol and other membrane lipids have been shown in various experimental systems to facilitate attachment of neurotransmitters to postsynaptic membranes (Fantini and Barrantes 2009). It has been demonstrated that decreased cholesterol levels (either endogenously (Sjogren, Csoregh et al. 2008; Paila, Tiwari et al. 2011) or with pharmaceutical reduction using HMG-CoA reductase inhibitors (Shrivastava, Pucadyil et al. 2010)) impair serotonin receptor signaling. Prior in vitro and in vivo studies are also consistent with impaired serotonin function in SLOS. For example, impaired serotonin receptor signaling was reported in cells treated with AY9944 (Paila, Murty et al. 2008). AY9944 is an inhibitor of DHCR7 and, thus, can be used to provide a pharmacological model of SLOS (Fliesler, Richards et al. 1999). In addition, Singh et al. (Singh, Paila et al. 2007) demonstrated, in vitro, that altered serotonin_{1A} receptor ligand binding is altered by 7DHC, and Waage-Baudet et al. (Waage-Baudet, Lauder et al. 2003) showed that the developing serotonergic system is altered in *Dhcr7* mutant embryos. Our current data further support the hypothesis that altered serotonin function may contribute to the abnormal cognitive and behavioral phenotype of SLOS.

Dietary cholesterol supplementation is currently used to treat SLOS patients. Although anecdotal reports suggest benefit with respect to behavioral manifestations of the disorder (reviewed in (Porter 2008)), given that cholesterol does not cross the blood-brain-barrier, it is not mechanistically clear how this could occur. Indirect mechanisms, such as altered neurosteroid production are possible; however, the single placebo-controlled trial that has tried to address this issue did not demonstrate any impact on behavioral manifestations of SLOS (Tierney, Conley et al. 2010). Although some of the neurological defects in SLOS are developmentally based, it is likely that functional abnormalities due to elevated 7DHC in neuronal synapses contribute to the behavioral phenotype. Developmental defects are likely not amendable to therapy, but treating functional deficits related to altered sterol biochemistry may be. Development of therapies that can improve the sterol biochemistry in the CNS of SLOS patients thus may prove beneficial. However, establishing clinical efficacy of therapeutic interventions in SLOS will be difficult because of the small number of patients and phenotypic heterogeneity. In the setting of a formal clinical trial, biomarkers can be useful tools to determine if a candidate drug may be having beneficial effects. As discussed above, decreased CSF 5HIAA and HVA levels in SLOS patients may reflect impaired synaptic vesicle formation which would be a functional defect that could respond

to therapies that improve the sterol biochemistry in SLOS. Furthermore, treatments designed to increase CSF levels of serotonin or dopamine may be beneficial.

Acknowledgments

Funding support: This work was supported by the intramural program of the National Institute of Child Health and Human Development and a Bench-to-Bedside award from the Office of Rare Diseases and the NIH Clinical Center. Autism Speaks, the Johns Hopkins Institute for Clinical and Translational Research (ICTR) which is funded in part by Grant Number UL1 TR 000424-06 from the National Center for Advancing Translational Sciences (NCATS) a component of the National Institutes of Health (NIH), and NIH Roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of the Johns Hopkins ICTR, NCATS or NIH. We would like to thank Richard Kelly for critical review of this manuscript. We would like to express our appreciation to the patients and families that participated in this study.

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Synopsis

Decreased cerebral spinal fluid serotonin and dopamine metabolite levels in Smith-Lemli-Opitz subjects provide insight into the pathology of this inborn error of cholesterol synthesis and provide a biomarker to monitor in experimental therapeutic trials.

Figure 1.

A) CSF levels of 5HIAA (filled squares) and HVA (filled circles) are significantly decreased compared to age appropriate reference ranges. B) There is a strong correlation ($r^2=0.74$, $p<0.0001$) between the degree of 5HIAA and HVA deficiency in individual subjects, suggesting a common underlying mechanism. CSF levels of 5HIAA do not correlate with either CSF cholesterol (C) or dehydrocholesterol (D) levels. CSF levels of HVA do not correlate with either CSF cholesterol (E) or dehydrocholesterol (F) levels.

Table 1

SLOS subjects and neurotransmitter metabolite levels

Subject	Gender	Age (years)	Severity Score	DHCR7 Genotype	5-HIAA (nmol/L) Baseline	Age Specific Ref Range (nmol/L)	HVA (nmol/L) Baseline	Age Specific Ref Range (nmol/L)
S02	F	4.42	11	p.C380Y/c.964-1G>C	62	66-338	135	218-852
S03	M	8.75	22	c.964-1G>C/p.T93M	56	66-338	122	218-852
S04	M	13.33	11	p.R242C/p.R450L	25	67-189	60	167-563
S05	M	8.92	11	c.964-1G>C/p.T289I	107	66-338	399	218-852
S06	M	10.58	6	c.964-1G>C/p.T289I	93	67-189	238	167-563
S07	M	7.08	11	c.964-1G>C/p.T93M	30	66-338	76	218-852
S08	M	9.75	28	p.W151X/p.T93M	73	66-368	174	218-852
S09	F	7.58	22	p.T154M/p.R443H	41	66-338	159	218-852
S10	F	12.0	17	p.R242C/p.W177R	87	67-189	282	167-563
S11	M	5.58	6	c.964-1G>C/p.A247V	101	66-338	202	218-852
S12	F	17.5	17	c.964-1G>C/p.R352W	106	67-140	192	145-324
S13	F	10.33	6	p.P51S/p.W151X	10	67-189	20	167-563
S15	M	4.75	6	p.T289I/c.964-1G>C	52	66-338	152	218-852
S16	F	5.42	22	p.T93M/p.R443C	96	66-338	139	218-852
S17	M	6.33	11	p.S169L/c.964-1G>C	73	66-338	200	218-852
S18	M	4.0	11	c.964-1G>C/p.V466M	90	66-338	208	218-852
S19	M	4.08	17	p.M1V/p.Q98X	68	66-338	224	218-852
S20	M	6.92	6	p.M1V/p.R446Q	110	66-338	308	218-852
S21	F	4.08	11	c.964-1G>C/p.Y462H	49	66-338	196	218-852
S22	M	13.25	22	p.W151X/p.V466M	92	67-189	160	167-563
S23	F	6.08	6	p.S192F/p.Y462H	18	66-338	87	218-852