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Oxidative stress, serotonergic changes and decreased ultrasonic vocalizations in a mouse model of Smith Lemli Opitz Syndrome

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Abstract

Smith Lemli Opitz syndrome is an inherited monogenic disorder in which mutations to the Dhcr7 gene lead to deficits in cholesterol synthesis. As a result, many patients suffer from gross physiological and neurological deficits. The purpose of this study was to identify a potential abnormal behavioral phenotype in a compound mutant mouse model for Smith Lemli Opitz disease ($Dhcr7^{\Delta 3–5/T93M}$) to further validate the model, and to provide potential targets for future therapeutic interventions. We also sought to identify some of the underlying changes in brain function that may be responsible for behavioral differences among groups. Dhcr7 compound mutant mice were smaller than their single mutant litter-mates. Both single and compound heterozygous mice made fewer ultrasonic vocalizations when separated from the dam which may suggest a communication deficit in these animals. Striking increases of the highly oxidizable 7-DHC were observed in the compound mutant mice. 7-DHC is the precursor to cholesterol and builds up due to decreased function of the mutated Dhcr7 enzyme. Additionally, several differences were noted in the serotonergic system including increased expression of the serotonin transporter (SERT) and increased uptake of serotonin by isolated synaptosomes. We propose that changes to the oxidative environment during development can have a significant impact on the development of serotonergic function and that this contributes to behavioral differences observed in the mutant mice.

Keywords

Smith Lemli Opitz Syndrome; ultrasonic vocalizations; serotonin; Oxysterols; Vitamin E; SERT; Mouse model; Dhcr7; antioxidant; development

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1. Introduction

Smith-Lemli-Opitz Syndrome (SLOS) is a monogenic neurodevelopmental disorder. It results from inherited mutations in both copies of the gene coding for 7-dehydrocholesterol reductase (Dhcr7), the last enzyme in the cholesterol biosynthesis pathway. Cholesterol is an essential component of cell membranes and neurotransmitter transporter regulation, and is highly enriched in the brain. A clinically-significant portion of SLOS patients (50–75%) present with Autism Spectrum Disorder behaviors (Nowaczyk & Irons, 2012, Sikora *et al.*, 2006). At present there is no cure for SLOS, and only moderately successful treatments for partial alleviation of some symptoms (Svoboda *et al.*, 2012).

In addition to cholesterol depletion, Dhcr7 dysfunction leads to accumulation of its highly reactive precursor 7–dehydrocholesterol (7-DHC). 7-DHC is the most oxidizable lipid identified to date, and its auto-oxidation leads to formation of over a dozen distinct oxysterols both *in vitro* and *in vivo* (Porter, 2000). Many of these are measurable both in human SLOS fibroblasts, and in the brain and other organs of Dhcr7 mutant mice, while only barely-present, or not measurable in control cells and animals (Xu *et al.*, 2009, Xu *et al.*, 2010, Xu *et al.*, 2011). Oxysterols also decrease viability of cultured neurons and neuronal cell lines indicating their potential role in neural damage (Korade *et al.*, 2010, Xu *et al.*, 2012).

The molecular pathophysiology underlying behavioral changes in SLOS and Autism Spectrum Disorder is poorly understood, but there is evidence for the involvement of the serotonergic (5-HT) system in SLOS and Autism in general (Posey *et al.*, 2006, Scott & Deneris, 2005, Waage-Baudet *et al.*, 2003). The most consistent neurochemical finding in Autism Spectrum Disorder is increased levels of blood platelet 5-HT (Cook & Leventhal, 1996). We propose that at least part of the abnormal behavioral phenotype observed in SLOS patients may be linked to aberrant development of the serotonergic system due to high levels of oxidative damage during a critical window of development.

A compound heterozygous mouse line carrying two different mutations derived from human SLOS cases ($Dhcr7^{\Delta 3–5/T93M}$ (Correa-Cerro *et al.*, 2006)) models many of the major physiological facets of the disease. These include decreased embryonic and post-natal survival rates, enlarged ventricles and two to three toe syndactyly. $Dhcr7^{\Delta 3–5/T93M}$ mice also have elevated tissue 7-DHC levels in cortex and midbrain although some improvement is seen with age (Correa-Cerro *et al.*, 2006, Marcos *et al.*, 2007, Meljon *et al.*, 2013). The compound mutant model is an important advance in the SLOS research field as it more accurately represents the clinical population, in which patients typically carry two different mutations, compared to earlier models that carried a single Dhcr7 mutation. Far more than one hundred different mutations of DHCR7 have been reported, but $DHCR7^{T93M}$ is one of the most common human missense mutations across multiple populations and usually presents with a reasonably mild SLOS phenotype (Correa-Cerro & Porter, 2005). Nevertheless, these mice have not yet been fully behaviorally phenotyped as juveniles or adults, data which will be critical in supporting the genotype-phenotype relationship.

We sought to identify abnormal behavioral outcomes in juvenile Dhcr7$^{\Delta 3–5/T93M}$ mice that may relate to the autism phenotype observed in patients. We therefore focused on ultrasonic communication in pups separated from the dam. We hypothesized that mutant mice would also show differences in serotonergic function as a result of oxidative damage by oxysterols during critical windows of serotonergic development.

2. Materials and Methods

Mice and breeding

Dhcr7$^{\Delta 3–5/WT}$ and Dhcr7$^{T93M/T93M}$ mice were originally obtained from Dr. Forbes D Porter, NICHD. These mice were back-crossed for >10 generations onto a C57bl/6J background. Homozygous Dhcr7$^{T93M/T93M}$ mice are viable and carry a point mutation in the T89 codon in mouse *Dhcr7* (single amino acid substitution - methionine for threonine) which is homologous to the T93 codon in the human gene (Correa-Cerro *et al.*, 2006). The second SLOS model carries a null disruption of *Dhcr7* incorporating a deletion of coding exons 3, 4, and 5 ($^{\Delta 3–5}$)(Wassif *et al.*, 2001). Homozygous Dhcr7$^{\Delta 3–5/\Delta 3–5}$ mice do not survive past birth. Initial breedings to obtain experimental parents were from Dhcr7$^{\Delta 3–5/+}$ X Dhcr7$^{\Delta 3–5/+}$, and Dhcr7$^{T93M/WT}$ X Dhcr7$^{T93M/WT}$ allowing for generation of wild-type mice for breeding. Dhcr7^{WT} wild-type (WT) controls are bred from wild-type littermates of the mutant breeders in order to match the background strain. Experimental SLOS mutant mice are bred by crossing Dhcr7$^{T93M/T93M}$ with Dhcr7$^{\Delta 3–5/WT}$. Litters are obtained from both Dhcr7$^{T93M/T93M}$ and Dhcr7$^{\Delta 3–5/WT}$ dams to control for potential influence from maternal genotype. Offspring are either Dhcr7$^{T93M/\Delta 3–5}$ (compound heterozygous) or Dhcr7$^{T93M/WT}$ (single heterozygous). Female mice were housed singly or in pairs following mating, until 48 hours prior to expected delivery at which point they were single housed. Pups remained with the dam until post-natal day 21–22 at which point they were weaned and/or euthanized. All animal housing and testing procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee. Both male and female offspring were tested on all measures. Behavioral testing took place during the light phase.

Experimental diets

Diets were designed using Research Diets, Inc. (New Jersey) for an earlier study (Harrison *et al.*, 2009a) and have previously been used with an alternative SLOS mouse model (Korade *et al.*, 2013a). The antioxidant diet (AOX, D04101103) contains α-tocopherol 400 IU/kg diet with vitamin C at 1 g/kg diet. The control diet (AIN-76A) contains 50 IU vitamin E provided in the standard vitamin mix and no vitamin C. In contrast, standard chow used in all other experiments (Lab Rodent Diet, 5001) contains no vitamin E or C.

Survival

Following timed matings, pregnant dams were single housed in clean cages beginning 2–3 days before expected delivery. Dams were checked daily, by 9 am, for signs of delivery. On postnatal days P0–P2 pups were counted and any dead pups (whole or partial) were removed and stored at −20° for genotyping. Survival rates are calculated from 7 litters from WT mice and 6 litters from mutant mice.

Weight

To avoid unnecessary handling pups were weighed on a gram scale only on days when behavioral tasks were scheduled (P7, P10 and P21). N=62 WT mice, N=59 Dhcr7$^{T95M/WT}$ and N=14 Dhcr7$^{\Delta 3–5/\ T95M}$ were tested at P7.

Ultrasonic vocalizations (USV)

USVs were measured at P7 (when vocalizations peak in C57Bl/6 mice (Scattoni *et al.*, 2009a)). Mice were briefly removed from the dam and placed individually in an insulated, sound-attenuating chamber. USVs were measured for 10 min. using an ultrasonic microphone and Avisoft software (Avisoft Bioacoustics, Germany). N=56 WT mice, N=48 Dhcr7$^{T95M/WT}$ and N=13 Dhcr7$^{\Delta 3–5/\ T95M}$ were tested at P7.

Nest building

Five grams of bedding material (~2 nestlets, Ancare, New York) was added to the cages on the day of birth. Nest quality was scored on a scale of 1 to 5, 24 and 48 hours later (based on (Deacon, 2006)).

Pup-retrieval

At P10, pups were removed from the home cage and placed on a warming pad. Three pups were placed in different corners of the home cage that did not contain the nest. The dam was returned to the cage and timed for latency to pick up each pup, and to return each to the nest. N=8 WT litters and N=5 Dhcr7 mutant litters were tested.

Activity

At P21 pups were tested in automated locomotor activity chambers for 30 min. (30cm x 30cm x 16cm; Med Associates Inc, VT). Lighting within the box is fixed at 32 Lux, with fans in each chamber providing a low level of standardizing background noise (approx. 75dB SPL). N=102 WT mice, N=52 Dhcr7$^{T95M/WT}$ and N=13 Dhcr7$^{\Delta 3–5/\ T95M}$ were tested.

Neuromuscular strength

Following activity measurements mice were tested for grip strength on the inverted screen task (2 trials)(Harrison *et al.*, 2008). The equipment was adapted for younger mice by affixing a smaller wire mesh to the screens.

Sterols

Cholesterol, desmosterol, lanosterol and 7-DHC Samples were obtained using Folch and/or specific lipid extraction protocols and run using HPLC-MS, with standards synthesized at Vanderbilt (Korade *et al.*, 2013b, Liu *et al.*, 2014). Samples from N=8 WT mice, N=7 Dhcr7$^{T95M/WT}$ and N=5 Dhcr7$^{\Delta 3–5/\ T95M}$ were tested.

Oxidative stress

Lipid peroxidation products were measured as malondialdehyde (thiobarbituric acid (Sigma Aldrich, USA) reactive substances) as published (Harrison *et al.*, 2009b). Samples from N=24 WT mice, N=20 Dhcr7$^{T95M/WT}$ and N=6 Dhcr7$^{\Delta 3–5/\ T95M}$ were tested.

Eicosanoids

F_2-isoprostanes and F_4-neuroprostanes were measured by the Vanderbilt Eicosanoid Core according to published methods (Milne *et al.*, 2007). Samples from N=11 WT mice, N=17 Dhcr7$^{T95M/WT}$ and N=4 Dhcr7$^{\Delta 3–5/\ T95M}$ were tested.

5-HT, dopamine and metabolites

Monoamines were measured in cortex using HPLC through the Vanderbilt Neurochemistry Core as described (Ye *et al.*, 2015). Samples from N=15 WT mice, N=19 Dhcr7$^{T95M/WT}$ and N=6 Dhcr7$^{\Delta 3–5/\ T95M}$ were tested.

SERT expression

Expression of SERT in midbrain was measured using Western blot with anti-5HTT antibody (Frontier Institute Co., Japan, diluted 1:2,000) with secondary antibody Anti-Guinea Pig IgG (A7289, 1:5,000, Sigma-Aldrich, USA) as described (Ye *et al.*, 2015). SERT expression was normalized to expression of β-actin (D35E4, 1:400 Santa Cruz Biotechnology, Dallas Texas) with Anti-Goat IgG (A5420, 1:5,000, Sigma-Aldrich, USA) as a secondary. Protein samples from N=6 WT mice, N=6 Dhcr7$^{T95M/WT}$ and N=5 Dhcr7$^{\Delta 3–5/\ T95M}$ were tested.

5-HT uptake in synaptosomes

Radiolabeled [^{3}H]-5-HT (50 μM) uptake into isolated synaptosomes (50 μg) from freshly dissected midbrain tissue was performed for 10 min at 37°C using a Brandel Cell Harvester, as previously described (Ye *et al.*, 2015). Results were calculated as moles/mg protein/min x10^{-16} with normalization to control animals within each of three separate experiments. Samples from N=5 WT mice, N=6 Dhcr7$^{T95M/WT}$ and N=1 Dhcr7$^{\Delta 3–5/\ T95M}$ were tested.

Statistical analyses

Whole litters were tested on behavioral measures because mice were not genotyped until after sacrifice at P21. Data were analyzed using SPSS 22.0 for Mac. Each variable was checked for normality and skew. Normally distributed data were analyzed using Univariate ANOVA with Fisher's LSD-corrected post hoc comparisons following a significant omnibus ANOVA. For data that violated assumptions of normality Kruskal-Wallis non-parametric analyses were used with individual Mann-Whitney analyses on pairs of data to establish differences among groups. P value was corrected by number of comparisons (i.e. α=0.05/3). Where comparisons were made between 2-groups only, t-tests were conducted (2-tail). Data were first analyzed with sex as a separate variable. No differences according to sex were detected so data were collapsed by genotype unless otherwise stated. For biochemical measures samples were selected pseudo-randomly from different litters according to sex and genotype.

Results and Discussion

Lower survival and body weight in mutant mice

Mendelian statistics suggest that litters should be comprised of 50% of each mutant genotype (Dhcr7$^{T93M/WT}$ and Dhcr7$^{T93M/\Delta 3–5}$). However, SLOS mutant mice, and in

particular Dhcr7$^{T93M/\Delta3–5}$ mice were significantly less likely to survive past postnatal day 2 both for primiparous and experienced dams (χ^2 = 29.3, P<0.001; χ^2 = 7.75, P<0.01, respectively, Fig. 1a). Dhcr7$^{T93M/\Delta3–5}$ mice were also smaller than their Dhcr7$^{T93M/WT}$ littermates at P7 (t(63)=2.80, P<0.01 Fig. 1b) and P21 (Mann-Whitney U=5.55=65.6, P<0.01, Fig. 1c). A similar smaller size and decrease from expected numbers of compound mutant pups was observed by Correa-Cerro et al. (Correa-Cerro *et al.*, 2006) and was attributed to maternal genotype (Dhcr7$^{T93M/T93M}$). We observed the same lower number of compound heterozygous pups from both Dhcr7$^{T93M/T93M}$ and Dhcr7$^{\Delta3–5/WT}$ dams. Direct comparisons were only made between Dhcr7$^{T93M/WT}$ and Dhcr7$^{T93M/\Delta3–5}$ littermates, and not WT mice to avoid the confounding factor of litter size. Sex differences were not observed at either age (Ps>0.6) so data were combined for both sexes.

Dhcr7 mutant mice make fewer and shorter calls when separated from the dam—Clear and valid behavioral and neuropathological targets help in understanding the etiology of disease and in testing potential therapies. USV is an early form of social communication that occurs between pups and dams, particularly in response to separation from the nest or physiological stress. We chose USV in juvenile mice as a task that has both construct and face-validity for autism spectrum disorders (Crawley, 2000) which is affected in other mouse models of autism spectrum disorders (Ey *et al.*, 2011). At P7, Dhcr7$^{T93M/\Delta3–5}$ and Dhcr7$^{T93M/WT}$ mice made significantly fewer USVs than WT following separation from the dam (Kruskal-Wallis χ= 10.62, P<0.01; individual Mann-Whitney U follow up analyses showed both genotypes were significantly lower than WTs at P<0.016 - α corrected for multiple (3) comparisons, Fig. 1d). The mean duration of calls was also shorter in the mutant mice, although this was only significant in Dhcr7$^{T93M/WT}$ pups ($F_{(2, 125)}$=4.62, P<0.05, Fig. 1e). Mouse USVs have been clearly demonstrated to be an effective means to elicit response from the dam, and to be altered in multiple mouse models of neurodevelopmental disorders (Scattoni *et al.*, 2009b). Alternative causes for decreased USVs in rodents include changes in temperature, olfactory and tactile information, and pain (Blumberg & Sokoloff, 2001, Hofer, 1996) that are part of the maternal-infant interaction, but are more a result of physiological than psychological modulation. Although all experimental conditions were identical for each of the genotypes, it is possible that the smaller Dhcr7$^{T93M/WT}$ pups were differentially affected by the different temperature in the test chamber compared to their nest, which could reflect an additional reason for decreased survival in these pups.

Juvenile mice were tested at P21 for activity and measures of neuromuscular strength. Total distance traveled in 20 mins. did not differ at this age ($F_{2, 161}$=2.33, P=0.10, Fig. 1f), and all mice spent a similar amount of time in the center versus the edge of the chamber during the first 5 mins. of testing (Kruskal-Wallis χ^2 =2.42, P=0.30, Fig. 1g). Given the smaller size of the compound mutant pups we had expected to see poorer performance on a test of neuromuscular strength. Performance on the inverted screen task did not vary according to genotypes (P>0.75), however, performance was poor in all genotypes at this age, with an overall mean latency to fall of 17.77 s ($\pm$ S.E.M. 1.42).

Maternal behaviors did not differ according to Dhcr7 genotype—It was not possible to breed WT and mutant litter-mates, and thus Dhcr7 mutant mice, but not WTs, were bred from dams that also carried at least one mutated copy of the Dhcr7 gene (either $Dhcr7^{\Delta 3–5/WT}$, or $Dhcr7^{T93M/T93M}$). We therefore included some measures of maternal behavior to establish that USV differences among pups were not due to differences in maternal treatment, or in physical differences among the genotypes, particularly given the smaller size of the mutant mice. Nest building did not differ according to dam genotype during the critical first 2 days post birth in which death rates were highest in mutant mice. All mice scored a minimum of 4 out of 5, indicating that >90% of nesting material had been used to build a clearly identifiable nest, although the nest may not have had all walls greater than the mouse's height (Deacon, 2006)(*data not shown*). We initiated pup retrieval testing to provide a measure of whether dams may be less responsive to calls from mutant genotypes. If that were the case, then lower call numbers could be a learned behavior through decreased reinforcement rather than resulting from a developmental difference. We observed no differences in latency to retrieve the first pup ($t(11)=0.64$, $P=0.54$, Fig. 1h) or to return all three pups to the nest ($t(11)=1.79$, $P=0.10$) at P10 according to genotype of the dam (WT, or $Dhcr7^{T93M/T93M}$ and $Dhcr7^{\Delta 3–5/WT}$; Fig. 1i). Pup genotypes were not established until sacrifice at P21–22 and pups were selected from the litter at random for retrieval task. Nevertheless, 40% of the first pups retrieved by mutant dams were $Dhcr7^{T93M/\Delta 3–5}$, indicating that it is unlikely that dams were less responsive to calls from these pups.

Overall these findings suggest that USV differences in the pups are not due to differences in maternal behaviors, or gross activity of physical differences in the compound heterozygous mice. We therefore sought to establish neurological differences that may have contributed to the effects observed.

Sterol and oxidative stress levels differed according to genotype

Cholesterol: A subpopulation of autism cases also have reduced cholesterol levels (Tierney *et al.*, 2006), which may be undiagnosed cases of SLOS, or at least share a common pathophysiological pathway in addition to sharing the disruption of serotonergic function. There was a trend towards decreased cholesterol in the cortex of $Dhcr7^{T93M/\Delta 3–5}$ mice ($F_{(2, 17)}=2.66$, $P=0.09$, Fig. 2a). Some correction in this model has been noted (Marcos *et al.*, 2007). It is therefore likely that the cholesterol deficit in $Dhcr7^{T93M/\Delta 3–5}$, and potentially $Dhcr7^{T93M/WT}$ mice would have been significant at P7 when USV was undertaken, although this was not measured explicitly. Lanosterol, which is an early precursor for cholesterol synthesis, did not differ among groups ($P=0.15$, *data not shown*). An additional role for the Dhcr7 enzyme is conversion of 7-dehydrodesmosterol to desmosterol. We showed a significant decrease in desmosterol in $Dhcr7^{T93M/\Delta 3–5}$ mice ($F_{(2, 17)}=17.78$, $P=0.001$, Fig. 2b), indicating that the mutations did indeed impact enzymatic function.

7-DHC: 7-DHC was used as an indirect measure of Dhcr7 enzyme activity, with higher levels indicating lower activity of the enzyme. 7-DHC levels were extremely low in all WT samples. 7-DHC increased significantly in $Dhcr7^{T93M/WT}$ mice (Kruskal-Wallis $\chi^2 = 16.71$, $P<0.001$, Fig. 2c), and even more so in $Dhcr7^{T93M/\Delta 3–5}$ mice. Given the highly oxidizable

nature of both 7-DHC and the oxysterols that are its breakdown products, we hypothesized that this accumulation may be the primary driver cause of neurological abnormalities, rather than cholesterol deficiency alone.

Oxidative stress: There is a strong oxidative damage component in SLOS, and antioxidant therapies are already in use in some populations. To show that the oxidative imbalance in SLOS is specific to, or at least highly dependent on 7-DHC, we assessed other markers of lipid peroxidation. MDA was significantly elevated in Dhcr7$^{T93M/WT}$ mice ($F_{(2, 48)}$=4.53, P=0.05, Fig. 2d) compared to WT (P<0.01), and also compared to Dhcr7$^{T93M/\Delta3–5}$ mice (P<0.05) indicating altered oxidative profile in those mice. MDA derives from the oxidation of arachadonic acid and thus the lower MDA levels in Dhcr7$^{T93M/\Delta3–5}$ mice more likely reflect a balance between increased oxidative stress and less of the starting material rather than a protected state in compound mutant mice compared to littermates. We sought to confirm this pattern of effects among the groups by measuring isoprostanes and neuroprostanes. Isoprostanes are also derived from arachadonic acid, but offer a more specific and stable marker of oxidative damage. Neuroprostanes are derived from docosahexanoic acid. Neither measure was significantly different among groups (F_2-Isoprostanes Kruskal-Wallis χ^2=2.04, P=0.36; F_4-Neuroprostanes, Kruskal-Wallis χ^2=0.29, P=0.87 Fig. 2e, f). Both arachadonic acid and docosahexanoic acid were decreased in brain an alternate knockout mouse model for SLOS, although decreases were only significant for docosahexanoic acid (*Dhcr7*$^{tm1Gst/J,}$ knockout) (Korade *et al.*, 2013b). In those mice there were no differences in isoprostanes, but a significant elevation of neuroprostanes. Our data support changes in the oxidative stress profile of this mouse model. However, future work should focus on the SLOS-specific oxidative damage mechanism relating to oxidized 7-DHC and downstream products (oxysterols).

Dhcr7 mutant mice exhibit changes in serotonin levels and uptake in midbrain

Serotonin (5-HT) and Biogenic Amines: The drug classes most frequently prescribed for behavioral management in autism spectrum disorders and SLOS include serotonergic modifiers such as SSRIs (selective serotonin reuptake inhibitors). Although beneficial in many cases of autism spectrum disorders, SLOS patients may respond differently to such drugs if connectivity or transporter expression varies in this population. It thus becomes critical to identify the specific deficit(s) in 5-HT brain system in order to tailor treatments to ameliorate behavioral problems.

At post-natal day 21 5-HT was increased in the cortex of Dhcr7$^{T93M/\Delta3–5}$ (Kruskal-Wallis χ^2 = 6.24, P<0.05, Fig. 3a) compared to WT mice. Detected levels of 5-HIAA, its breakdown product, did not differ among groups, which was reflected in a lower 5-HIAA/5-HT ratio in these mice (Kruskal-Wallis χ^2 = 1.70, P=0.43; Kruskal-Wallis χ^2 = 6.64, P<0.05, respectively, Fig. 3b,c). No differences among groups were found in dopamine, nor any of its breakdown products (DOPAC, HVA, 3-MT), nor in the DOPAC/DA ratio (Ps>0.15).

If the increased serotonin was related to altered uptake in these animals, and if this finding translates to humans, it could be a critical factor in establishing how a patient may respond

to SSRIs and similar drugs. SERT activity was increased following 1 month of treatment with cholesterol lowering Simvastatin in hypercholesterolemic patients (Vevera *et al.*, 2005). SERT expression in midbrain was elevated in mutant mice, although this was only significant in Dhcr7$^{T93M/\Delta3–5}$ mice ($F_{(2, 14)}$=3.65, P<0.05) and not Dhcr7$^{T93M/WT}$ mice (P=0.053, Fig. 3d). In order to assess whether this related to functional differences in 5-HT uptake we prepared synaptosomes from freshly dissected midbrain and cortex. There was increased uptake of radiolabeled 5-HT in synaptosomes from midbrain of Dhcr7$^{T93M/WT}$ mice compared to wild-type animals (t(9)=2.91, P<0.05, Fig. 3e). The same difference was not observed in cortical preparations (t(9)=2.0, P=0.08, Fig. 3f). To minimize inter-experimental differences, data are provided as uptake relative to WT control. Data range for midbrain was 2.99 (WT) to 7.31 (Dhcr7$^{T93M/\Delta3–5}$) moles/mg protein/min x10^{-16}, and for cortex was 3.86 (WT) to 7.75 (Dhcr7$^{T93M/\Delta3–5}$) moles/mg protein/min x10^{-16}. Statistical analyses were not performed on synaptosome extracts from Dhcr7$^{T93M/\Delta3–5}$ mice because only a single animal was born in the three litters that were available for this assay. Nevertheless, uptake in midbrain synaptosomes the Dhcr7$^{T93M/\Delta3–5}$ mouse studied was even greater than that observed in the Dhcr7$^{T93M/WT}$ mice.

Antioxidant supplementation to pregnant females supports normal weight in offspring but does not alter sterol profile in brain

Vitamin C is critical for recycling of vitamin E to prevent α-tocopherol radicals contributing to pro-oxidant rather than antioxidant reactions. Although not an essential supplement for mice which can synthesize their own in liver (unlike humans), during pregnancy vitamin C synthesis increases in mice to match needs of fetuses (Corpe *et al.*, 2010). Its addition to the diet helps protect against additional strain on the maternal environment by oxidative stress due to her own, or the fetal genotypes. We have previously shown that these diets significantly increase vitamin E (α-tocopherol) in both brain and liver at P0 in heterozygous *Dhcr7*$^{tm1Gst/J}$ mutant mice (Korade *et al.*, 2013a), and more importantly, decrease 7-DHC-derived oxysterols in brain. α-tocopherol inhibited accumulation of DHCEO, an oxysterol derived from 7-DHC in cultured fibroblasts carrying SLOS mutations (Korade *et al.*, 2013a). Antioxidant supplementation also modified expression of a number of genes involved in lipid biosynthesis in these cells. We therefore, sought to understand whether an antioxidant diet enriched with α-tocopherol and ascorbic acid (Harrison *et al.*, 2009a, Korade *et al.*, 2013a) provided to the pregnant dam could be sufficient to modify some of the abnormalities observed in the Dhcr7$^{T93M/WT}$ and Dhcr7$^{T93M/\Delta3–5}$ mice.

Multiparous dams were used to improve odds of survival among Dhcr7$^{T93M/\Delta3–5}$ mice. Two out of 14 pups died in antioxidant-supplemented dams (14.2%, from 2 litters), which was not different from control-supplemented mice (20.9% from 9 litters)(P>0.05). We noted a gain in weight in antioxidant-supplemented Dhcr7$^{T93M/\Delta3–5}$ mice compared to non-supplemented Dhcr7$^{T93M/\Delta3–5}$ mice (t(13)=3.05, P<0.01, Fig. 4a), which was not observed in Dhcr7$^{T93M/WT}$ (t(48)=1.82, P=0.075) or WT mice (t(68) = 0.32, P=0.75 *not shown*). The diet did not alter the highly elevated levels of 7-DHC in the brain in Dhcr7$^{T93M/\Delta3–5}$ (Fig. 4b).

Conclusions

Our data suggest that it may be important to shift the focus of SLOS research to recognize specific neurological changes to the oxidative environment in these patients and the potential effect on serotonergic function. We propose that decreasing the oxidative damage that occurs during critical windows of development could impact serotonergic system development and thus have a direct impact on neural health and behavior. These relationships, along with safety of supplement-type interventions during pregnancy must also be studied in human populations. If found to be effective, then early interventions to prevent damage could minimize deficits and optimize quality of life for patients and families.

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Abbreviations

USV	ultrasonic vocalizations
SLOS	Smith Lemli Opitz Syndrome
Dhcr7	7-dehydrocholesterol reductase gene
5-HT	5-hydroxytryptamine, serotonin
5-HIAA	*5*-hydroxyindoleacetic acid
P#	postnatal day (where P0 is day of birth)
SERT	serotonin transporter

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Highlights

- Dhcr7 mutant pups make fewer ultrasonic calls when separated from the dam
- Serotonin levels, turnover, and uptake are all altered in Dhcr7 mutant pups
- SERT expression is increased in Dhcr7 compound mutant mice

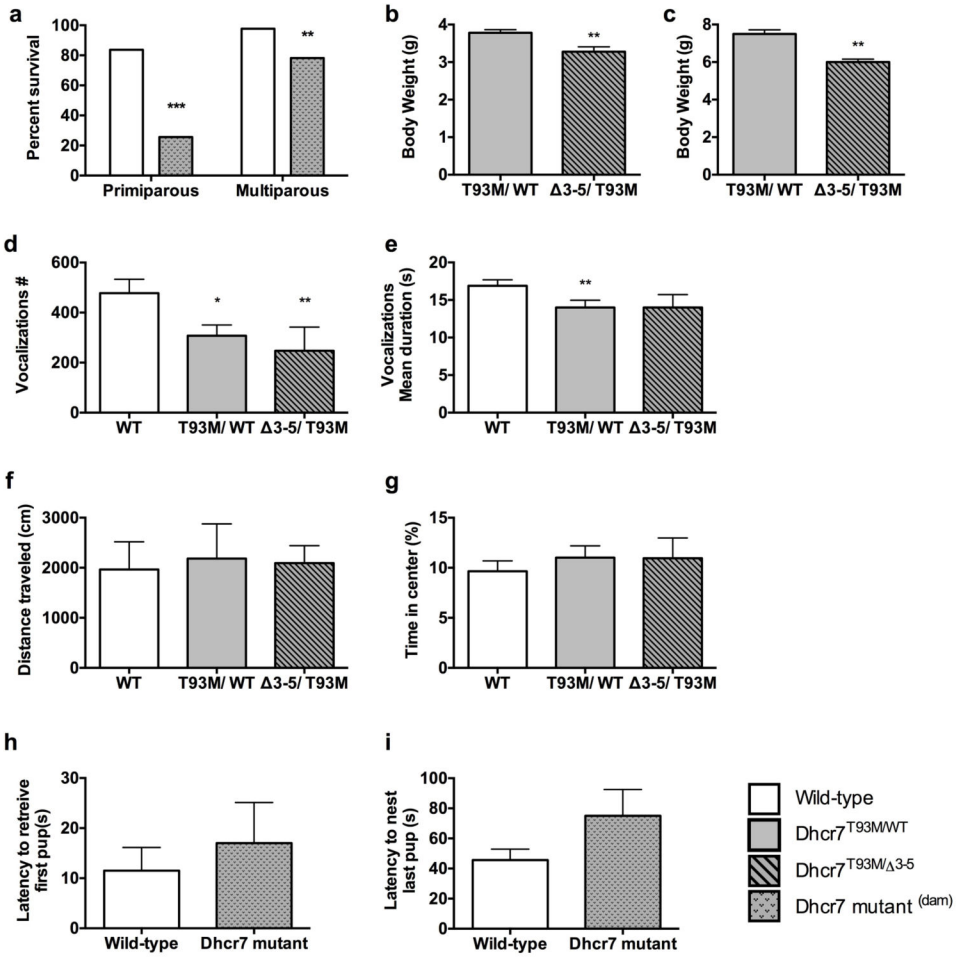

Figure 1. Lower survival and disrupted ultrasonic communication in Dhcr7 mutant mice

(a) Survival of Dhcr7 mutant mice is lower than WTs for both primiparous and multiparous females. Data from a total of 43 births from 6 litters for WT mice, 43 births from 7 litters for Dhcr7 mutant mice. (b,c) Body weight was lower in Dhcr7$^{T93M/\Delta3–5}$ compared to Dhcr7$^{T93M/WT}$ at both post-natal day 7 and 21. (d) Both Dhcr7$^{T93M/\Delta3–5}$ and Dhcr7$^{T93M/WT}$ mice made fewer ultrasonic vocalizations (USVs) that WT mice. (e) Calls by mutant mice were also shorter than those made by WT mice which was significant in Dhcr7$^{T93M/WT}$ mice (P<0.01), but not Dhcr7$^{T93M/\Delta3–5}$ (P=0.077). (f, g) There was no difference among groups in the distance travelled in the activity chambers, or percent time spent in the central portion of the chamber. (b–g) WT: N=67–102, Dhcr7$^{T93M/WT}$: N=39–52, Dhcr7$^{T93M/\Delta3–5}$: N=8–13 mice per experiment. (h, i) WT dams were not significantly faster to retrieve, or re-nest their pups compared to Dhcr7 mutant dams. WT 8 litters, Dhcr7 5 litters tested. *, **, *** P<0.05, 0.01, 0.001 different from WT control (a, d, e), or different from Dhcr7$^{T93M/WT}$ littermate controls (b, c).

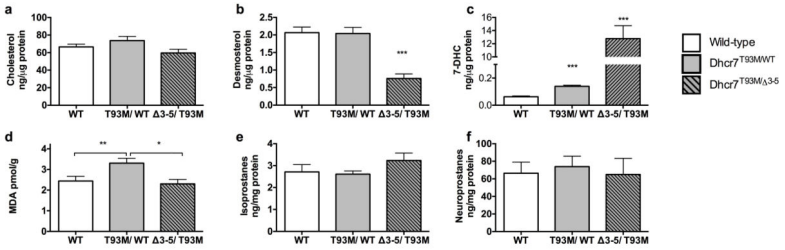

Figure 2. Cholesterol and oxidative stress measures

(a) Cholesterol was slightly decreased in cortex of Dhcr7$^{T93M/\Delta3–5}$ mice at P21, but this was not significant. (b) In contrast, a striking decrease was seen in desmosterol in Dhcr7$^{T93M/\Delta3–5}$ mice. (c) The effect of the Dhcr7 mutations were further visible through elevated 7-DHC in both Dhcr7$^{T93M/WT}$ and Dhcr7$^{T93M/\Delta3–5}$ mice. (d) MDA was slightly increased in Dhcr7$^{T93M/WT}$ compared to wild-types, but not in compound mutant littermates. Neither (e) F_2-Isoprostanes or (f) F_4-Neuroprostanes differed among groups. (a–f) WT: N=8–24, Dhcr7$^{T93M/WT}$: N=7–20, Dhcr7$^{T93M/\Delta3–5:}$ N=4–6 mice per experiment. *, **, *** P<0.05, 0.01, 0.001 different from both other genotypes unless otherwise marked.

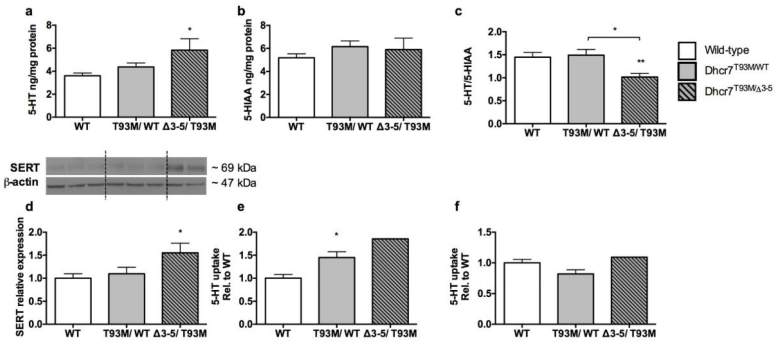

Figure 3. Serotonergic abnormalities in Dhcr7 mutant mice

(a) There was significantly greater 5-HT detected in Dhcr7$^{T93M/\Delta3–5}$ compared to WT controls. (b, c) 5-HT breakdown product 5-HIAA levels did not differ but the 5-HT/5-HIAA ratio was much lower in Dhcr7$^{T93M/\Delta3–5}$ mice. (d) SERT expression in midbrain was also elevated in Dhcr7$^{T93M/\Delta3–5}$ mice. A representative Western Blot image shows bands for SERT at approximately 75 kDa, and for β-actin at approximately 47 kDa. Bands are shown above the relevant bars for each genotype in the bar chart. (e, f) Uptake of radiolabelled 5-HT into synaptosomes was significantly greater in Dhcr7$^{T93M/WT}$ mice compared to WT in midbrain but not cortex. An even greater increase was observed in midbrain synaptosomes in the Dhcr7$^{T93M/\Delta3–5}$ mouse that was tested, with no additional change in cortex. (a–c) WT: N=15, Dhcr7$^{T93M/WT}$: N=19, Dhcr7$^{T93M/\Delta3–5:}$ N=6 mice per experiment. (d–f) N=5–6 for all genotypes except (e, f) Dhcr7$^{T93M/\Delta3–5}$ N=1. * P<0.05 different from WT, unless otherwise marked.

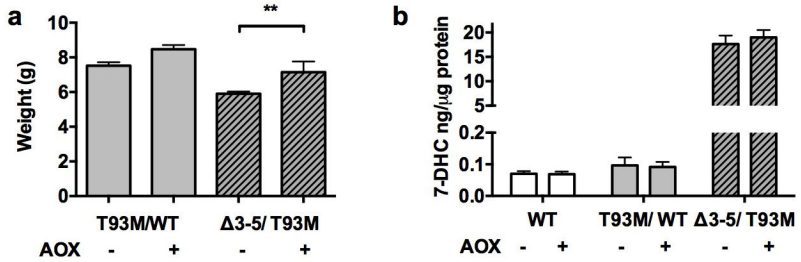

Figure 4. Antioxidant (AOX) supplements improve birth weight in Dhcr7$^{T93M/\Delta3–5}$ mice

(a) Dhcr7 mutant mice from dams fed AOX supplemented diets were heavier than mice of the same genotype from control diet-fed dams (Dhcr7$^{T93M/WT:}$ AOX-N=43, AOX+ N=7; Dhcr7$^{T93M/\Delta3–5}$ AOX-N=11, AOX+ N=4). (b) 7-DHC in brain did not alter in P21 pups according to the diet fed to dams (Ns=3–4 per group).