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Research paper

High levels of mitochondrial DNA are associated with adolescent brain structural hypoconnectivity and increased anxiety but not depression

Olga Tymofiyeva[a,*,1], Eva Henje Blom[b,c,1], Tiffany C. Ho[b,d], Colm G. Connolly[b], Daniel Lindqvist[b,f], Owen M. Wolkowitz[b], Jue Lin[e], Kaja Z. LeWinn[b], Matthew D. Sacchet[g], Laura K.M. Han[h], Justin P. Yuan[a], Sarina P. Bhandari[a], Duan Xu[a], Tony T. Yang[b]

[a] Department of Radiology & Biomedical Imaging, University of California San Francisco, United States
[b] Department of Psychiatry and Weill Institute for Neurosciences, University of California San Francisco, United States
[c] Department of Clinical Sciences/ Child, and Adolescent Psychiatry, Umeå University, Umeå, Sweden
[d] Department of Psychology, Stanford University, United States
[e] Department of Biochemistry and Biophysics, University of California San Francisco, United States
[f] Lund University, Faculty of Medicine, Department of Clinical Sciences Lund, Psychiatry, Lund, Sweden
[g] Department of Psychiatry and Behavioral Sciences, Stanford University, United States
[h] Department of Psychiatry, Amsterdam Neuroscience, VU University Medical Center, GGZ inGeest, Amsterdam Public Health research institute, Amsterdam, The Netherlands

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ABSTRACT

Background: Adolescent anxiety and depression are highly prevalent psychiatric disorders that are associated with altered molecular and neurocircuit profiles. Recently, increased mitochondrial DNA copy number (mtDNA-cn) has been found to be associated with several psychopathologies in adults, especially anxiety and depression. The associations between mtDNA-cn and anxiety and depression have not, however, been investigated in adolescents. Moreover, to date there have been no studies examining associations between mtDNA-cn and brain network alterations in mood disorders in any age group.

Methods: The first aim of this study was to compare salivary mtDNA-cn between 49 depressed and/or anxious adolescents and 35 well-matched healthy controls. The second aim of this study was to identify neural correlates of mtDNA-cn derived from diffusion tensor imaging (DTI) and tractography, in the full sample of adolescents.

Results: There were no diagnosis-specific alterations in mtDNA-cn. However, there was a positive correlation between mtDNA-cn and levels of anxiety, but not depression, in the full sample of adolescents. A subnetwork of connections largely corresponding to the left fronto-occipital fasciculus had significantly lower fractional anisotropy (FA) values in adolescents with higher than median mtDNA-cn.

Limitations: Undifferentiated analysis of free and intracellular mtDNA and use of DTI-based tractography represent this study's limitations.

Conclusions: The results of this study help elucidate the relationships between clinical symptoms, molecular changes, and neurocircuitry alterations in adolescents with and without anxiety and depression, and they suggest that increased mtDNA-cn is associated both with increased anxiety symptoms and with decreased fronto-occipital structural connectivity in this population.

1. Introduction

Adolescent anxiety and depression are both highly prevalent with numerous long-term negative health consequences (Polanczyk et al., 2015). To advance understanding of these disorders and improve prevention and treatment approaches, comprehensive research at multiple levels of analysis is required, as suggested in the Research Domain Criteria (RDoC) framework (Insel et al., 2010). There is, however, a persistent gap in research that would span more than two levels of analysis. Specifically, the relationship between clinical symptoms (self-reports), molecular changes (intracellular markers), and neurocircuitry alterations in adolescents with anxiety and depression remains largely unclear.

* Correspondence to: Department of Radiology & Biomedical Imaging, University of California, San Francisco, 1700 4th St., Byers Hall Suite 102, San Francisco, CA 94158, United States.
E-mail address: Olga.Tymofiyeva@ucsf.edu (O. Tymofiyeva).
[1] These authors contributed equally to this work.

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One promising intracellular marker that has been studied in adults with depression and anxiety is the mitochondrial DNA copy number (mtDNA-cn) in blood cells or in saliva. An increase of mtDNA-cn has been found in some studies to be associated with stress and several psychopathologies in adults, especially anxiety and depression (Cai et al., 2015; Tyrka et al., 2016; Wang et al., 2017; Edwards et al., 2016) but this marker has not yet been studied in adolescents, near the time of onset of these disorders.

Depending on the cell and tissue type, each human cell contains between several hundred and over a thousand mitochondria, each carrying 2–10 copies of mtDNA (Robin and Wong, 1988). The human mtDNA is a double-stranded, closed circular molecule encoding 37 genes essential for normal mitochondrial functioning. It has been found that the relative content of mtDNA increases with age (Lee et al., 1998) and that the mtDNA content is positively correlated with the level of oxidative stress (Lee et al., 2000), although non-trivial relationships between blood levels of circulating cell-free mtDNA and an antioxidant enzyme have also been observed (Lindqvist et al., 2018). Mitochondrial biogenesis serves the energy demands of the cell, and one possible explanation of the observed correlations within cells is that the increase in mtDNA content may be a mechanism to prepare cells to respond to endogenous or exogenous oxidative stress through cell-cycle arrest (Lee et al., 2000). An increase in mtDNA-cn may therefore indicate a feedback mechanism and may serve as an index of compensatory mitochondrial biogenesis in the case of increased oxidative stress levels.

Mitochondria are also increasingly recognized as a signaling platform involved in fundamental events in the formation and plasticity of neuronal circuits (Cheng et al., 2010). Specifically, there is evidence that changes in mitochondrial bioenergetics can have major effects on the brain circuitry (Picard and McEwen, 2014). Disturbances in mitochondrial functions and signaling have been suggested to play roles in impaired neuroplasticity and neuronal degeneration in Alzheimer's disease, Parkinson's disease, stroke, and psychiatric disorders (Cheng et al., 2010).

Whereas studies of the association between mtDNA-cn and psychiatric disorders remain sparse, studies linking mtDNA-cn and brain structure and function are completely lacking. A large body of literature demonstrated abnormal brain circuitry in various psychiatric disorders (reviewed by Cao et al., 2015; Rubinov and Bullmore, 2013; Griffa et al., 2013; Menon, 2011), including fronto-striatal white matter hypoconnectivity in both adult (Korgaonkar et al., 2014) and adolescent depression (LeWinn et al., 2014; Tymofiyeva et al., 2017). It remains unclear whether mtDNA-cn is linked to structural brain connectivity. Understanding alterations of mitochondrial biogenesis and neural correlates of these alterations in psychiatric disorders may help establish more effective therapeutic strategies for these disorders and thus lead to better outcomes for affected individuals.

To fill this significant knowledge gap and help address these unanswered questions, the ***first aim*** of this study was to compare salivary mtDNA-cn between clinically depressed and/or anxious adolescents and well-matched healthy controls. Salivary mtDNA was chosen for two reasons: 1) saliva is much easier to collect in adolescents than blood and 2) the largest and most impactful study in the field by Cai et al. used saliva to assess mtDNA content in humans, while their animal work produced comparable results for blood and saliva (Cai et al., 2015). Based on previous studies in adults linking higher mtDNA-cn with Major Depressive Disorder (MDD) (Cai et al., 2015; Tyrka et al., 2016; Wang et al., 2017; Edwards et al., 2016) and anxiety disorders (Tyrka et al., 2016), we hypothesized a higher mtDNA-cn in adolescents with MDD, with or without comorbid anxiety, compared to well-matched healthy controls. The ***second aim*** was to identify structural neurocircuitry correlates of mtDNA-cn in adolescents using diffusion tensor imaging (DTI). Based on the previously reported negative effects of disturbances in mitochondrial bioenergetics on the brain circuitry (Picard and McEwen, 2014; Cheng et al., 2010), we hypothesized that high mtDNA-cn would be associated with white matter hypoconnectivity.

2. Materials and methods

2.1. Participants and clinical information

The Institutional Review Boards at the University of California San Diego (UCSD), University of California San Francisco (UCSF), Rady Children's Hospital in San Diego, and the County of San Diego approved this study. All participants in the study provided written informed assent and their parent(s) or legal guardian(s) provided written informed consent in accordance with the Declaration of Helsinki.

The study protocol, recruitment procedures, clinical and diagnostic assessments, and inclusion/exclusion criteria have been previously described (LeWinn et al., 2014; Tymofiyeva et al., 2017) and are included here in brief. A subset of 84 postpubertal adolescents from Tymofiyeva et al. (2017), who had both usable DTI and mtDNA-cn data were included in this study. This dataset consisted of 49 adolescents with MDD according to the DSM-IV (mean age at time of scan 16.1 ± 1.3yrs. [13.1–17.9], 27 females) and 35 well-matched healthy controls (HC) (mean age at time of scan 16.1 ± 1.4yrs. [13.2–17.9], 22 females). Five of the depressed subjects were using psychotropic medication (three subjects - Selective Serotonin Reuptake Inhibitors (SSRIs), one subject - quetiapine and amitriptyline, and one - dexmethylphenidate) at the time of the scanning and 44 were unmedicated. The MDD and HC groups were well matched on age, gender, Tanner pubertal stage, Hollingshead socioeconomic status, and intelligence. Details of the groups' characteristics can be found in Table 1.

Table 1
Demographic and clinical characteristics of the study participants.

	MDD[a]	HC[a]	Statistic[b,c]	p value	Effect Size (95% CI)	Signif.
Number of participants in final analysis (n)	49	35				
Gender (M / F)	22 / 27	13 / 22	$\chi^2(1.00) = 0.24$	0.63		
Age at time of scan (years)	16.1 ± 1.3 (13.1–17.9)	16.1 ± 1.4 (13.2–17.9)	t(70.76) = 0.11	0.91	g = 0.02 (−0.41; 0.46)	
Hollingshead Socioeconomic Score	40 ± 39 (11–70)†	29 ± 23 (0–66)†	W = 1014	0.15	PS = 0.38 (0.26; 0.51)	
Tanner Score	4 ± 0.5 (3–5)†	4 ± 0.5 (3–5)†	W = 976	0.26	PS = 0.3 (0.2; 0.43)	
Wechsler Abbreviated Scale of Intelligence	101.4 ± 12.4 (77–129)	104.8 ± 9.1 (84–125)	t(81.95) = −1.45	0.15	g = −0.32 (−0.76; 0.12)	
Children's Depression Rating Scale (Standardized)	71.9 ± 8.6 (55–85)	33.9 ± 5.8 (30–55)	t(81.77) = 24.28	< 0.001	g = 5.32 (4.40; 6.25)	***
Reynolds Adolescent Depression Scale (Standardized)	65.4 ± 8.5 (35–78)	41.9 ± 7.7 (30–56) [1]	t(75.33) = 13.10	< 0.001	g = 2.87 (2.25; 3.49)	***
Multidimensional Anxiety Scale for Children (Standardized)	59.9 ± 9.8 (32–78) [2]	41.8 ± 9.2 (26–61) [2]	t(71.54) = 8.48	< 0.001	g = 1.86 (1.34; 2.38)	***

Abbreviations: MDD, Major depressive disorder; HC, healthy control; M, male; F, female. CI, Confidence Interval; SD, standard deviation; g, Hedge's g; '***' p < .001.

[a] Mean ± SD (min–max) or median ± interquartile range (min - max) if indicated by †. The optional number in [] indicated the number of missing data points.

[b] Statistic: W, Wilcox rank sum test; χ^2, χ^2 test for equality of proportions; *t*, Student's *t*-test.

[c] Statistics for clinical scales refer only to participants included in the final analysis.

2.2. Clinical and self-report assessments

Depression severity was assessed for each participant using both a clinician-administered scale, the Children's Depression Rating Scale-Revised (CDRS-R) (Poznanski and Mokros, 1996), and a self-report scale, the Reynolds Adolescent Depression Scale (RADS-2) (Reynolds, 2002). As part of the original study, the CDRS-R scores were used to further differentiate the study groups: controls with CDRS-R *T*-scores higher than 54 and MDD participants with *T*-scores lower than 55 were excluded. Because the CDRS-R was used, in part, to determine group assignment, the RADS-2 scores were used in the present study for group and correlation analyses. The RADS-2 standardized scores and empirically derived clinical cutoff scores provide an indication of the clinical severity of the individual's depressive symptoms. This 30-item self-report measures the four basic dimensions of depression: Dysphoric Mood, Anhedonia/Negative Affect, Negative Self-Evaluation, and Somatic Complaints, whereas the depression total score represents the overall severity of depressive symptomatology. Anxiety symptoms were assessed with the Multidimensional Anxiety Scale for Children (MASC) (March et al., 1997). The MASC consists of four main scales, three of which have subscales: Physical Symptoms (Tense/Restless, Somatic/Autonomic), Social Anxiety (Humiliation/Rejection, Performance Fears), Harm Avoidance (Perfectionism, Anxious Coping), and Separation/Panic (March, 1998). The MASC also yields a Total Scale score and an Anxiety Disorder Index score to identify youth who may meet criteria for an anxiety disorder.

2.3. mtDNA data acquisition, processing and analysis

The mtDNA data were extracted from saliva collected in an Oragene DNA kit (DNA Genotek, Kanata, Ontario, Canada). The personnel who performed the assay received deidentified samples and were blind to all other measurements. Detection of a 69 bp fragment of the ND1 gene in mtDNA (nucleotides 3485–3553) and an 87 bp fragment of RNase P (TaqMan® Copy Number Reference Assay, human, RNase P, cat# 4403328, Life Technologies) by a TaqMan multiplex assay was used to determine the relative copy number of mtDNA per diploid nuclear genome. This assay was adapted from previous published methods (He et al., 2002). The primer and probe sequences for ND1 were:

ND1-forward	5′-CCCTAAAACCCGCCACATCT-3′
ND1-reverse	5′-GAGCGATGGTGAGAGCTAAGGT-3′
ND1-FAM *probe*	5′ **FAM**-CCATCACCCTCTACATCACCGCCC-**TAMRA**-3′

The reaction contained 12.5 ng of genomic DNA, 100 nM of ND1 probe, 300 nM of ND1-forward primer and ND1-reverse primer each, 1 × RNase P copy number Reference Assay, 1 × LightCycler® 480 Probe Master (Roche, cat# 04902343001) in a 10ul reaction. All samples were run in triplicate wells in 384-well plates in a Roche LightCyler 480. PCR conditions were 95 °C 10 min for 1 cycle; 45 cycles of 95 °C 10 s, 60 °C 30 sec, 72 °C 1 s with data acquisition at 72 °C. Crossing point (Cp) for each well was derived by the LightCycler 480 program using the second derivation method. Relative copy number per diploid genome was calculated by the following formula: Relative mtDNA copy number = POWER{2, (Cp_{ND1}-CP_{RNaseP})}*2. The interassay coefficient of variation (CV) was 3.4%.

2.4. MRI data acquisition and network construction

To address the second goal of the study, structural brain connectivity was assessed using MRI connectomics approach that allows for non-invasively mapping and analyzing brain networks. The acquisition, preprocessing, network construction and statistical analyses (Network-Based Statistic, NBS) steps were similar to our previous study (Tymofiyeva et al., 2017) and are briefly described below.

The data were collected using a 3 T MRI system (MR750, GE Healthcare, Milwaukee, Wisconsin, USA) at the UCSD Center for Functional Magnetic Resonance Imaging (CFMRI). High-resolution anatomical T1-weighted images were acquired using a fast spoiled gradient recalled (SPGR) pulse sequence (TR/TE = 8.1/3.17 ms, flip angle = 12°, slice thickness = 1 mm, FOV = 250 × 250 mm, 256 × 256matrix, 0.98 × 0.98 × 1 mm voxels). The diffusion-weighted images were acquired using a dual spin echo, single-shot echo-planar imaging (EPI) sequence, 30 directions, b-value = 1500 s/mm^2, TR/TE = 7200/86.5 ms, FOV = 180 × 180 mm, 96 × 96matrix, 1.875 × 1.875 × 2.5 mm voxels, two averages.

The T1-weighted images were bias-field-corrected, skull-stripped, and transformed to MNI152 space using an affine transform in FSL (Smith et al., 2004). A quality assurance step was performed on DTI data as previously described (LeWinn et al., 2014). DTI reconstruction and deterministic whole-brain streamline fiber tractography were performed using the Diffusion Toolkit (Wang et al., 2007) with Fiber Assignment by Continuous Tracking (FACT) and a threshold angle of 35°.

Cerebral segmentation into 90 regions of interest (ROIs) was performed in the DTI space using the Automated Anatomical Labeling (AAL) atlas (Tzourio-Mazoyer et al., 2002) and intermediate registration to T1-weighted images in MNI space. The ROIs were dilated by one voxel and used as network nodes. Connections between AAL ROIs were calculated using the average fractional anisotropy (FA) along the streamlines as weights. The FA-weighted connections were stored as a 90 × 90 connectivity matrix, in which each row/column corresponded to a distinct node (brain ROI).

2.5. Statistical analyses

The statistical analyses were performed using IBM SPSS Statistics software (version 25). Outlier analysis was performed using Tukey's method (Tukey, 1977) and extreme cases of mtDNA-cn with values > 3 times the interquartile range (IQR) were removed. Two models were tested. In the first model, mtDNA-cn was modeled as a function of the diagnostic group (MDD vs HC). Since the Shapiro-Wilks test of normality was significant for both groups, MDD and HC, we used a non-parametric approach, specifically, the independent-samples Mann-Whitney *U* test. Accounting for age was not considered necessary after linear regression analysis showed no significant relationship between age and mtDNA-cn in either of the groups, MDD or HC.

Because there was no statistical indication that the distribution of mtDNA differed between the two groups, and because we were interested in assessing depressive and anxiety symptoms in a continuous, not only dichotomous, manner, we pooled the two groups together and performed the remaining analyses in the full sample. Bivariate correlations were performed between mtDNA-cn and depressive and anxiety symptoms in the full sample.

In the second model tested in this study, structural brain connectivity was modeled as a function of mtDNA-cn (high-mtDNA-cn vs low mtDNA-cn groups, using median split in the full sample). To assess edge-wise differences in the connectivity matrices between the low- and high-mtDNA-cn groups, we utilized the NBS approach implemented in Matlab (Zalesky et al., 2010). Alpha threshold was set at the default value of 0.05. NBS also requires a choice of the primary threshold by the user, which can lead to differences in the resulting topologies. However, the control of family-wise error rate (FWER) is guaranteed irrespective of the threshold choice (Zalesky et al., 2010). We performed a *t*-test with 5 000 permutations and the strictest primary threshold chosen experimentally.

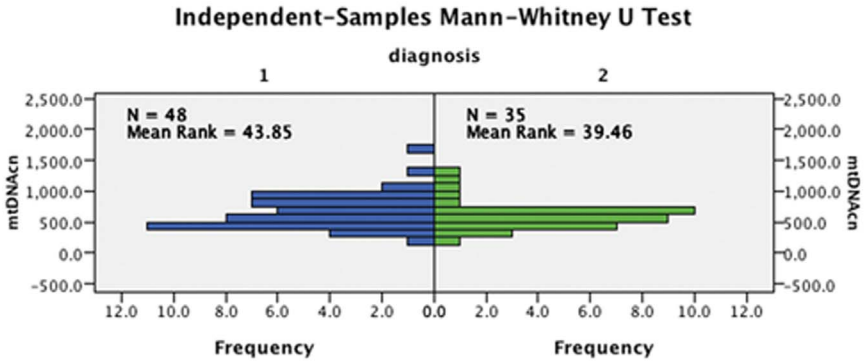

Fig. 1. Results of the independent-samples Mann-Whitney *U* test: there was no significant difference in mtDNA-cn between MDD (1) and HC (2) groups ($p = .412$).

3. Results

The MDD and HC adolescent groups showed the expected significant differences in levels of depression and anxiety with the MDD group having greater depression and anxiety on all scales (CDRS-R, RADS-2, MASC) (all p < .001; see Table 1). The MDD and HC groups did not significantly differ on age, gender, pubertal stage, IQ, and socioeconomic status.

3.1. mtDNA-cn analysis

Based on the outlier analysis using Tukey's method, one adolescent with MDD was removed from the analysis. The remaining 83 adolescents' datasets were included in the analysis. There was no significant difference in mtDNA-cn between MDD and HC groups (independent-samples Mann-Whitney *U* test resulted in $p = .412$, Fig. 1).

In the pooled sample of the adolescents, both parametric and non-parametric correlations between mtDNA-cn and depressive symptoms, RADS-2 *T*-scores, were not significant: Pearson correlation r = 0.080, $p = .474$, N = 82; Spearman's rho = 0.046, $p = .682$, N = 82 (one HC subject did not have RADS-2 scores available). However, the correlation between mtDNA-cn and anxiety symptoms measured using MASC *T*-scores was significant: Pearson correlation r = 0.254, $p = .024$, N = 79; Spearman's rho = 0.226, $p = .046$, N = 79 (two HC and two MDD subjects did not have MASC scores available). At the same time, MASC was highly correlated with RADS-2: Pearson correlation r = 0.770, $p < .001$, N = 79. We report Pearson correlation coefficient in addition to Spearman correlation coefficients, since Pearson correlation coefficient is most commonly used and demonstrates robustness to departures from normality in larger datasets (Bishara and Hittner, 2012).

Since we found a statistically significant correlation result for mtDNA levels and anxiety, we further explored different subscales of the MASC scale (Table 2). The analyses revealed that the correlation was largely driven by Somatic/Autonomic and Humiliation/Rejection subscales, both showing Spearman's rho > 0.2 (Table 2). In this case, we only report the Spearman's rho, because we are not testing specific hypotheses but rather look for the largest contributing factor. Correction for multiple comparisons is not needed, since no hypothesis testing is performed in this case.

Table 2
Non-parametric correlations (Spearman's rho) of MASC subscales *T*-scores with mtDNA-cn in 78 adolescents.

	Correlation Coefficient	*p* (2-tailed)
MASC Total	0.226	0.046
Physical Symptoms	0.209	0.064
Tense/Restless	0.188	0.096
Somatic/Autonomic	0.238	0.034
Harm Avoidance	0.046	0.690
Perfectionism	−0.054	0.636
Anxious Coping	0.077	0.503
Social Anxiety	0.227	0.045
Humiliation/Rejection	0.239	0.034
Performance Fears	0.122	0.283
Separation/Panic	0.127	0.264
Anxiety Disorder Index	0.242	0.032

Removing medicated subjects, however, resulted in a non-significant correlation between mtDNA-cn and anxiety (Pearson r = 0.181, $p = .122$, N = 74; Spearman's rho = 0.145, $p = .217$, N = 74) (Fig. S1). The medicated and non-medicated subjects differed significantly both with respect to anxiety levels ($t = -2.069$, $p = .042$, independent samples *t*-test assuming equal variances) and mtDNA-cn ($t = -2.067$, $p = .042$, independent samples *t*-test assuming equal variances). An additional mediation analysis indicated that the effect of medication on mtDNA-cn may be mediated by anxiety (the direct effect of medication status on mtDNA-cn became non-significant, beta std. = 0.173, $p = .127$, when adding MASC to the linear regression, whereas the MASC effect remained close to significant, beta std. = 0.215, $p = .06$). This suggests that the loss of significance when removing the medicated subjects from the analysis is likely due to the decreased N.

Since there was no statistically significant group difference in mtDNA-cn, we pooled MDD and HC adolescents together and split them into high-mtDNA and low mtDNA groups (using median split), in order to examine, whether their brains differed with respect to structural connectivity.

The results of the NBS analysis at the threshold of 3.4, p < .05, and 1 000 permutations are presented in Fig. 2. The results were confirmed with the false discovery rate (FDR) analysis. In particular, significantly lower FA was observed in adolescents with high (above-median) mtDNA-cn in the following connections: between the orbital part of the left superior frontal gyrus (L. Frontal_sup_orb) and the left Heschl's gyrus (t = 3.49), between the orbital part of the left middle frontal gyrus (L. Frontal_mid_orb) and the left Heschl's gyrus (t = 3.43), between the left olfactory cortex (L. Olfactory) and the left Heschl's gyrus (t = 3.41), between the right olfactory cortex (R. Olfactory) and the left Heschl's gyrus (t = 3.41), and between the left cuneus cortex (L. Cuneus) and the left Heschl's gyrus (t = 3.51).

Fig. 3 shows an example participant's tractography streamlines going through the L. Heschl's gyrus, which demonstrate correspondence to the white matter track known as the fronto-occipital fasciculus (FOF).

After removing the medicated subjects, the NBS analysis produce results similar to those listed above for the full dataset: hypoconnected links between L. Frontal_sup_orb and the left Heschl's gyrus (t = 3.51), between the L. Frontal_mid_orb and the left Heschl's gyrus (t = 3.42), between the L. Olfactory and the left Heschl's gyrus (t = 3.42), and between the L. Cuneus and the left Heschl's gyrus (t = 3.50).

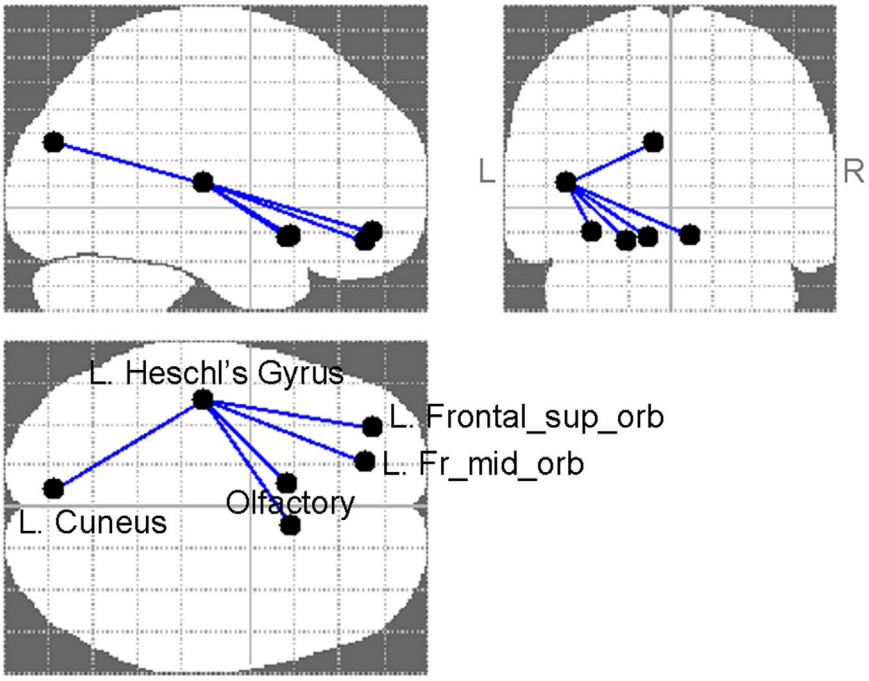

Fig. 2. NBS results at the primary threshold of 3.4, $p < .05$, 1000 permutations, depicting hypoconnected edges in the high-mtDNA group; confirmed with false discovery rate (FDR) analysis.

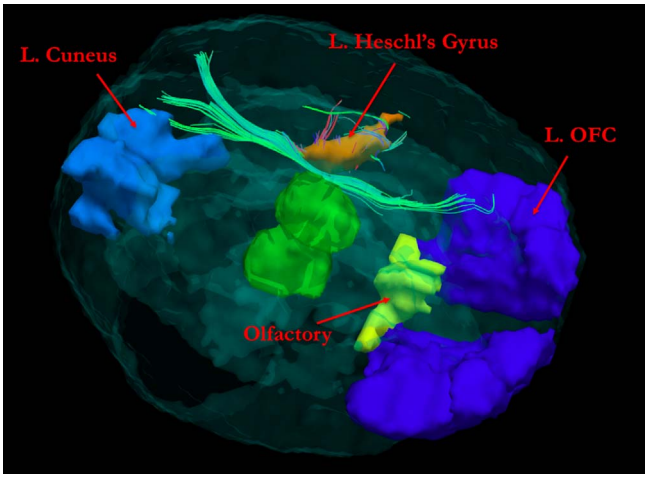

Fig. 3. Example of study participant's tractography streamlines going through the L. Heschl's Gyrus, largely corresponding to the fronto-occipital fasciculus. The thalamus is displayed in the center of the brain for a reference. L. OFC: left orbitofrontal cortex.

4. Discussion

Our first hypothesis regarding high levels of mtDNA copy number being associated with adolescent depression was based on certain previous studies in adult MDD (Cai et al., 2015; Tyrka et al., 2016; Wang et al., 2017; Edwards et al., 2016) and it was not confirmed. Interestingly, in a recent longitudinal study by Verhoeven *et al.*, the authors found no evidence for an association between depressive symptoms and mtDNA-cn in a community-based sample of depressed adults (either between-person or within-person) (Verhoeven et al., 2017). Moreover, a decrease of mtDNA-cn was observed in adult MDD in another study (Chang et al., 2015). It is important to consider the developmental aspect of this molecular marker. Depressive illness is often a chronic and recurrent disorder, and mtDNA copy number has been shown to positively correlate with the duration of the longest episode (Edwards et al., 2016). One can therefore speculate that the inconsistent findings of increased mtDNA-cn in adult, but not adolescent depression, may be explained by the effect of accumulated oxidative stress during the comparatively longer duration of depressive illness in adults. This might also be a reason why He et al. (2014) did not find any association between leukocyte mtDNA-cn in blood and MDD in young adults. The severity of symptoms might also play a role and recent studies found significantly higher mtDNA-cn in suicide attempters (Lindqvist et al., 2016) and completers compared to controls (Otsuka et al., 2017).

Notably, we observed a significant positive correlation between mtDNA-cn and levels of anxiety, but not depression, in a mixed group of adolescents, which was—in exploratory analyses—particularly driven by Somatic/Autonomic and Humiliation/Rejection subscales. This finding can also potentially explain the discrepancy with the published literature on mtDNA in adult depression, as we describe here. A recent study by Steenkamp *et al.* showed a positive correlation between peripheral oxidative stress and anxiety, but not depression symptoms, in physically healthy, medication-free individuals with MDD (Steenkamp et al., 2017). Specifically, they found that higher plasma levels of F2-isoprostanes and glutathione disulfide (GSSG) were associated with higher anxiety scores. The continuous relationships between severity of anxiety symptoms and oxidative stress marker levels showed small to medium effect sizes. If anxiety but not depression is associated with oxidative stress, which in turn may lead to increased mtDNA-cn, this could explain our findings in adolescents. At the same time, symptoms of generalized and social anxiety are known to be highly comorbid with depression symptomatology (Hettema, 2008) and, therefore, previous mtDNA-cn findings may have been mis-attributed to depression rather than anxiety. This highlights the importance of mapping biomarkers onto dimensional symptoms in addition to categorical DSM diagnoses (Steenkamp et al., 2017).

It should be noted that the correlation between mtDNA-cn and anxiety in the adolescent subjects in our study was non-significant when the five medicated subjects were removed from the sample. The medicated and non-medicated subjects differed significantly both with respect to anxiety levels and mtDNA-cn. Our additional analysis indicated that the effect of medication on mtDNA-cn may be mediated by anxiety, suggesting that the loss of significance is likely due to the decreased sample size. From a psychopharmacological perspective, it

needs to be mentioned that the major categories of drugs to treat affective disorders are known to impact mitochondrial function, and new-generation anti-depressive medications have preferential action on mitochondrial metabolism (Adzic et al., 2016). Future studies are needed to exclude the possibility that the effect of increased mtDNA-cn in anxious and depressed subjects is driven by antidepressant medication.

Our second aim was to identify network-level neural correlates of mtDNA-cn across all subjects. We used white matter microstructural properties, particularly fractional anisotropy (FA), as a proxy for structural connectivity between brain regions. FA has been widely used in studies of neuroplasticity, reflecting alterations in white matter fiber organization (including axon branching, sprouting, packing density, axon diameter, fiber crossing and the number of axons), as well as changes in myelin thickness and morphology (Zatorre et al., 2012). Since FA is impacted by multiple factors listed above (and some of them, such as crossing fibers, can misleadingly decrease FA without connectivity decrease), this metric cannot be used as a quantitative measure of brain connectivity (Jones et al., 2013). Nevertheless, impressive and useful qualitative results are continually obtained using FA, for example, in a recent neuroplasticity study with learning (Schlaffke et al., 2017).

In our study, we observed a significant difference in FA-weighted brain networks between adolescents with high and low mtDNA-cn. Specifically, those with mtDNA-cn above the median displayed weaker fronto-occipital connections in the left hemisphere compared to those with mtDNA-cn below the median. The finding persisted after excluding medicated subjects. It augments the finding in a sample partially overlapping with the sample of this study, showing that adolescent MDD was associated with the right fronto-striatal white matter hypoconnectivity (Tymofiyeva et al., 2017).

From previous studies of mitochondrial genetics it is known that dysfunctional mtDNA can influence plasticity of neuronal circuits (Picard and McEwen, 2014). In primates, even subtle changes in mitochondrial bioenergetics have shown major effects on the brain, which at least in part seems to be caused by their influence on synaptic transmission. It is not yet known whether these findings may be extended to humans (Picard and McEwen, 2014). In our study, fronto-occipital connections in the left hemisphere, with L. Heschl's gyrus as an intermediate node, showed difference between the high- and low-mtDNA-cn groups. The fronto-occipital fasciculus (FOF), also known as the inferior fronto-occipital fasciculus (IFO) is a large white matter tract connecting the frontal, temporal and occipital lobes. It constitutes one of the major efferent and afferent neuronal projections to the frontal lobes and connects the prefrontal cortex with auditory and visual association cortex in the temporal lobe (Kier et al., 2004). Heschl's gyrus contains the primary auditory cortex and is involved in hearing, which is commonly impaired in patients with mtDNA defects (Chinnery et al., 2000). It is possible that, since fronto-temporo-occipital connections develop more slowly than other regions (Lebel et al., 2008), they are particularly vulnerable to early life stress and therefore they are the ones showing the difference between the high- and low-mtDNA-cn groups in our study. The vulnerability of IFO to different forms of stress is supported by previous studies. For example, in a study by Huang et al., adolescents exposed to childhood maltreatment had significantly lower FA values in the left IFO (Huang et al., 2012). Bergamino et al. (2015) recently applied a free-water correction algorithm to female MDD patients' DTI data, which revealed low-FA clusters in the left IFO of the patients compared to controls, with significant correlations of DTI measures with reported stress levels. Finally, patients with obsessive compulsive disorder (OCD), which is associated with considerable anxiety, displayed structural hypoconnectivity of the inferior fronto-occipital fasciculus bilaterally, that correlated with symptom severity and neuropsychological performance (Garibotto et al., 2010).

Based on the results of this study and previous literature, we would like to suggest a tentative model (Fig. 4) that describes relationships between different units of analysis as suggested by the Research Domain Criteria (RDoC) (Insel et al., 2010). The units of analysis considered here include clinical symptoms (self-reports), molecular changes, and neurocircuitry alterations. According to this model, the environmental impact (such as childhood trauma) can play a causal role in depressive and anxiety symptoms (Hovens et al., 2010; Li et al., 2016), which themselves represent a form of chronic stress. Chronic stress promotes oxidative stress throughout the body (Aschbacher et al., 2013). Specifically, anxiety but not depression is reportedly associated with increased levels of oxidative stress (Steenkamp et al., 2017), which in turn leads to increased mtDNA-cn as a compensatory feedback mechanism (Lee et al., 2000). In this model, the disturbance of the mtDNA-cn negatively affects brain plasticity (Picard and McEwen, 2014) and leads to white matter hypoconnectivity in distinct brain regions, which may represent neurocircuitry that is vulnerable to stress during brain development (namely, the fronto-temporo-occipital circuit that develops more slowly than other regions (Lebel et al., 2008)). Other interpretations of the results are possible, which presuppose different chains of events. For example, inherited interpersonal polymorphic differences in mtDNA (haplogroups) and other common mtDNA mutations may be modifying physiological responses to psychosocial stressors and in this way mediating the association between stress exposure and later mental illness (Picard et al., 2015). In this view, stress-reactive axes are under mitochondrial regulation, whereby mitochondrial dysfunction alters the perceived physiological severity of various stressors (Picard et al., 2015). To represent this alternative model, Fig. 4 would need to be modified, in which the "mtDNA-cn" block would be placed between the "Environmental factors" and "Anxiety and depression symptoms" blocks. Being preserved across tissues, mitochondrial bioenergetics could be playing the role of systemic neuroendocrine modulator, explaining why some individuals show hypothalamic-pituitary-adrenal (HPA) hyperresponsiveness to psychological stress (Picard et al., 2015). This "filtered" stress would then lead to neural remodeling and altered fronto-temporo-occipital connectivity.

Several methodological limitations should be taken into account when interpreting our findings. First, in this study saliva was collected in the stabilizing solution in the Oragene kit, which lysed the cells, so the relative copy number of mtDNA per diploid nuclear genome was a mixture of the intracellular mtDNA and free mtDNA in saliva. Future research should include separate analyses of free and intracellular mtDNA, as important differences have been observed between the two quantities (Lindqvist et al., 2018). Second, a DTI-based tractography method was employed in this work to reconstruct structural brain networks. Although DTI is most widely used, it has a limited capacity for resolving the fiber crossing issue and may result in misleading information about fiber tracts orientation (Farquharson et al., 2013). High-order reconstruction methods may be better at resolving complex fiber crossings (Tuch et al., 2002), however, even these more

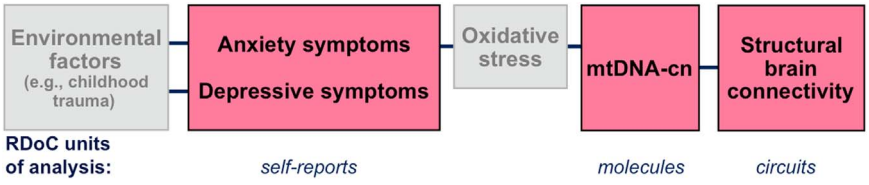

Fig. 4. The hypothesized model of adolescent depression and anxiety with multiple units of analysis. The hypothesis of oxidative stress mediating the link between anxiety symptoms and mtDNA-cn has not been tested in this study (the light gray box).

sophisticated tractography methods do not consistently show superior sensitivity and specificity (Thomas et al., 2014).

In summary, we did not observe differences in the mtDNA copy number between adolescents with clinical depression and well-matched healthy controls, although such differences were previously detected in some studies in adults. However, we observed a significant positive correlation between mtDNA-cn and levels of anxiety in a mixed group of adolescents. Notably, our study also suggests that there is a difference in white matter brain networks between adolescents with high versus low mtDNA-cn, specifically in the fronto-occipital connections of the left hemisphere. Since this study is the first to report these findings, our results will need to be confirmed in future studies. The results of our study highlight the importance of mapping biomarkers onto dimensional symptoms in addition to categorical DSM diagnoses and investigating the relationships between different units of analysis: molecular changes, neurocircuitry alterations, and clinical symptoms. We hope that our findings will help guide the design of future studies and contribute to improved prevention and treatment of adolescent anxiety and depression.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jad.2018.02.024.

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