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Cortical and Subcortical Glutathione Levels in Adults with Autism Spectrum Disorder

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Increased oxidative stress has been postulated to contribute to the pathogenesis of autism spectrum disorder (ASD). However, reports of alterations in oxidation markers including glutathione (GSH), the major endogenous antioxidant, are indirect, coming from blood plasma level measurements and postmortem studies. Therefore we used in-vivo 3 Tesla proton magnetic resonance spectroscopy ([1H]MRS) to directly measure GSH concentrations in the basal ganglia (BG) and the dorsomedial prefrontal cortex of 21 normally intelligent adult males with ASD and 29 controls who did not differ in age or IQ. There was no difference in brain GSH between patients and controls in either brain area; neither did GSH levels correlate with measures of clinical severity in patients. Thus [1H]MRS measures of cortical and subcortical GSH are not a biomarker for ASD in intellectually able adult men. ***Autism Res*** *2016, 9: 429–435.*
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Keywords: autism; magnetic resonance spectroscopy; glutathione; oxidative stress; redox

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by repetitive behaviors and impairments in reciprocal social interactions and communication [American Psychiatric Association, 2013], affecting about 1% of the population [Baron-Cohen et al., 2009].

The aetiology of ASD remains unclear, though various genetic, environmental and immunological factors are thought to influence the development of the disorder [Deth, Muratore, Benzecry, Power-Charnitsky, & Waly, 2008]. Among these, oxidative stress—particularly that arising from toxic environmental exposures—has been proposed to play a central role in its pathogenesis [Chauhan & Chauhan, 2006; Deth et al., 2008; Deth, 2013; Theoharides, Kempuraj, & Redwood, 2009]. This "Redox Hypothesis" is supported by reports of increased markers of oxidative stress and lipid peroxidation in individuals with ASD, including elevated red blood cell nitric oxide, decreased plasma levels of antioxidant proteins, and an imbalance between the reduced and oxidised forms of glutathione (GSH) [Chauhan, Chauhan, Brown, & Cohen, 2004; Kern & Jones, 2006].

GSH is the major endogenous cellular antioxidant. It protects against lipid peroxidation and oxidative stress, by maintaining the balance between production and removal of reactive oxygen species (ROS) [Shimizu et al., 1998]. Lower levels of GSH and higher levels of its oxidized metabolite, GSSG, have been reported in the plasma of individuals with ASD and this has been suggested to indicate greater oxidative stress [Frustaci et al., 2012; Geier et al., 2009; James et al., 2004; Main, Angley, O'Doherty, Thomas, & Fenech, 2012]. However, it is unclear whether this decrease in peripheral GSH also occurs in the brain. Decreased GSH, increased GSSG, and a decreased GSH:GSSG ratio have been reported in the cerebellum and temporal cortex of subjects with ASD post-mortem

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[Chauhan, Audhya, & Chauhan, 2012]. However, post-mortem studies are limited by the quality of protein preservation [Ferrer, Martinez, Boluda, Parchi, & Barrachina, 2008], therefore GSH concentrations in the ASD brain also need to be researched in vivo.

Brain GSH can be measured in vivo using the technique of proton magnetic resonance spectroscopy ([1H]MRS). This approach has been applied to individuals with various neuropsychiatric disorders using a 3T scanner. For instance, temporal cortex GSH levels have been reported to be higher in people with psychosis, whereas no difference in brain GSH has been found in those with bipolar disorder [Godlewska, Yip, Near, Goodwin, & Cowen, 2014; Lagopoulos et al., 2013; Wood et al., 2009]. However to date, no-one has examined [1H]MRS measures of GSH in ASD.

Therefore, in this study, we compared brain GSH in unmedicated adult men with ASD and healthy controls of similar age and IQ using [1H]MRS. [1H]MRS spectra were acquired in the basal ganglia (BG) and in the dorsomedial prefrontal cortex (DMPFC). These regions were chosen because they have been consistently reported to have both structural abnormalities and functional deficits (relevant to the core symptoms) in ASD (e.g. see [Haznedar et al., 2006; Hollander et al., 2005; Horder et al., 2013; Schmitz, Daly, & Murphy, 2007]. For instance, anomalies in the BG are thought to be responsible for repetitive behaviors [Hollander et al., 2005; Naaijen, Lythgoe, Amiri, Buitelaar, & Glennon, 2015], while frontal network deficits have been implicated in social symptoms [Bernhardt et al., 2014; Watanabe et al., 2012; Wicker et al., 2008]. We tested the hypotheses that men with ASD have lower GSH than controls and that this would correlate with the severity of ASD symptoms [assessed using the Autism Observation Schedule (ADOS) [Lord, Rutter, DiLavore, & Risi, 1999] and the Autism Diagnostic Interview—Revised (ADI) [Lord, Rutter, & Le Couteur, 1994] for current and past symptoms respectively]. As co-morbid symptoms of anxiety, depression and ADHD are common in ASD [Matson & Cervantes, 2014; Mazzone, Ruta, & Reale, 2012], we also explored potential correlations between GSH levels and symptoms rated using the State-Trait Anxiety Inventory (STAI) [Ferreira & Murray, 1983], the Beck depression inventory (BDI) [Beck, Ward, Mendelson, Mock, & Erbaugh, 1961] and the Barkley self-reported childhood and adulthood scales (BSSR-Child and BSSR-Adult) [Barkley & Murphy, 1998], respectively.

Materials and methods

Participants

Twenty one male participants with ASD and 29 healthy men who did not differ in IQ or age were included in this study. Participants were aged 18–50. All participants were right handed and had not been taking psychoactive medication for at least six weeks prior to the study. Full scale IQ was assessed by a trained researcher using the Wechsler Abbreviated Scale of Intelligence [Rudie et al. 2013]. Only participants with $IQ > 80$ were included in this study. Participants with ASD were recruited from the Behavioural Genetics Clinic at the Maudsley Hospital, a specialist adult autism diagnostic clinic. All had been diagnosed with autism by experienced psychiatrists according to the ICD-10 criteria (2009). The diagnosis was confirmed using the ADI and/or ADOS. We excluded individuals with a comorbid psychotic illness, epilepsy, a history of head injury, or a genetic disorder (e.g., Fragile X syndrome) known to be associated with ASD. Symptoms of anxiety, depression and attention deficit and hyperactivity disorder (ADHD) were measured using the STAI [Ferreira & Murray, 1983], the BDI and the BSSR-Child and BSSR-Adult scales respectively. All participants provided written informed consent to participate. This study was approved by Essex 2 National Research Ethics Committee.

[1H]MRS Data Acquisition and Processing

[1H] MRS data were acquired on a 3T GE HDx MRI scanner (GE Medical Systems, Milwaukee, WI, USA). A structural MRI scan for subsequent voxel positioning was acquired using a 3D fast inversion-recovery prepared gradient echo acquisition with $TI = 450$ ms, $TR = 7$ ms, $TE = 2.8$ ms, matrix $= 256 \times 256$ over a 240×240 mm field of view, giving a 0.9375×0.9375 mm in plane voxel size, 124×1.1 mm slices (partitions).

[1H]MRS spectra were acquired using point-resolved spectroscopy (PRESS) [Bottomley, 1987]. One voxel ($20 \times 20 \times 15$ mm^3) was placed in the left basal ganglia (BG), including the head of the caudate, the anterior putamen, and the internal capsule. Another voxel ($16 \times 24 \times 20$ mm^3) was placed in the left DMPFC (see Fig. 1). PRESS parameters were: $TR = 3000$ ms, $TE = 30$ ms. One hundred twenty-eight averages were collected in the BG voxel and 96 in the DMPFC voxel. [1H]MRS spectra were processed using LCModel software version 6.3-0I [Provencher, 1993].

Data were analysed with a basis set optimized for the detection of glutathione, which also included the following metabolites: alanine, aspartate, creatine, gamma-aminobutyric acid (GABA), glutamine, glutamate, GSH, glucose, glycerophosphocholine, phosphocholine, phosphocreatine, myo-inositol (mI), lactate, N-acetyl-aspartate (NAA), N-acetyl-aspartylglutamate (NAAG), scyllo-inositol, and taurine. However, only GSH was considered here.

Spectra were first reviewed visually to verify that spectra were not qualitatively abnormal. Poorly fitted metabolite estimates, defined as Cramer-Rao lower

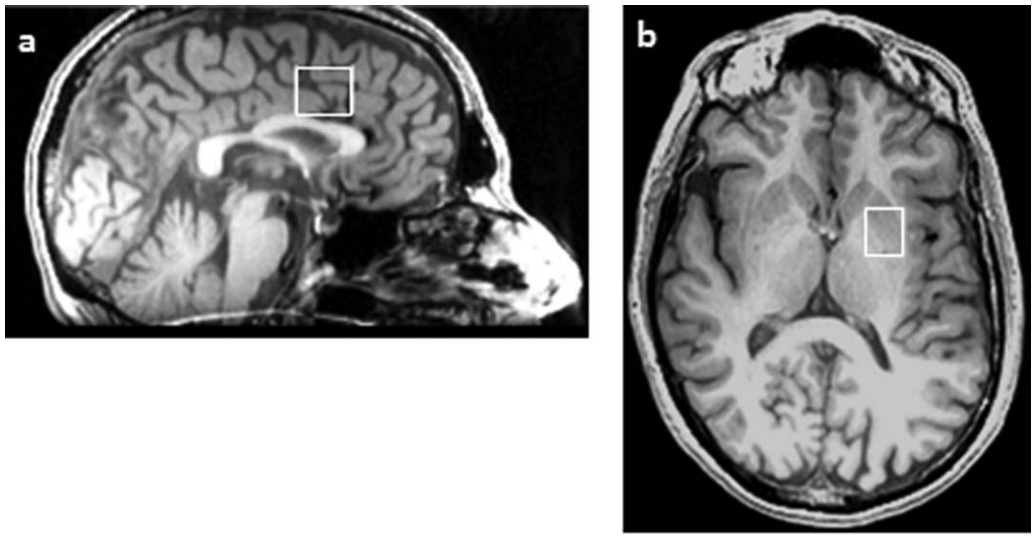

Figure 1. Example of the location of the proton magnetic resonance spectroscopy voxel in the dorsomedial prefrontal cortex (a), and in the basal ganglia (b).

bounds (CRLB) >20%, were excluded from further analysis.

Calculation of GSH Concentrations

To control for inter-individual differences in voxel tissue composition, the proportion of grey matter (GM), white matter (WM), and cerebrospinal fluid (CSF) in each voxel was calculated. This method provides a useful alternative to reporting metabolite concentrations as ratios to Creatine content [Brooks et al., 2001; Gussew, Erdtel, Hiepe, Rzanny, & Reichenbach, 2012]. The structural MRI was segmented into GM, WM and CSF using Statistical Parametric Mapping software (SPM2; http://spm.ion.ucl.ac.uk) and percent tissue class of each voxel was calculated using in-house software as previously described [Horder et al., 2013]. Raw metabolite values were corrected for CSF as follows:

$$GSH_{corrected} = GSH_{Raw}/(1-Proportion_{CSF})$$

Where $Proportion_{CSF}$ ranged between 0 and 1, and was calculated individually for each voxel. Raw metabolite values corrected for CSF are referred to as corrected GSH concentrations in the rest of this article.

Statistical Analysis

All data were analysed in IBM SPSS 21. Group differences in demographic variables and symptom severity were determined using independent sample *t*-tests. To investigate potential differences in GSH between groups, we used a general linear model with corrected GSH concentrations as the dependent variable, group as a between subjects factor, and age as covariate, to account for any GSH variation with age [Currais & Maher, 2013; Maurya & Rizvi, 2010].

The relationship between GSH and the severity of current ASD symptoms measured using the three subscales of the ADOS, of past ASD symptoms (three subscales of the ADI) and of anxiety, depression and ADHD symptoms (STAI, BDI, BSSR child and adult scales) was assessed using Pearson correlation coefficient or Spearman's rank correlation coefficient, depending on the data distribution. To account for multiple comparisons in these analyses, we used a "family wise" error correction. The *P*-value threshold of 0.05 was divided by 3 for analyses of correlations with three subscales of the ADOS; it was also divided by 3 for analyses of correlations with three subscales of the ADI; and it was divided by 4 for analyses of correlations with four comorbid symptom ratings.

Results

Participants

There was no significant difference in age or IQ between the ASD and control groups. As expected, the participants with ASD scored higher than controls on the questionnaire measures of depression, anxiety, and attention-deficit disorder symptoms (all $F > 4.7$, all $P < 0.04$). Participant characteristics are summarised in Table I. After excluding spectra where the uncertainty (% CRLB) of the GSH estimates were greater than 20% the sample size was 13 patients and 19 controls for the DMPFC voxel, and 16 patients and 18 controls for the BG voxel.

Glutathione

There was no significant effect of group on corrected GSH concentrations in either voxel ($F < 0.9$, $P > 0.1$);

neither was there a main effect of age, or an interaction between group and age. The results were unchanged when GSH was examined as a ratio to total Creatine (all $P > 0.3$). Mean corrected GSH concentrations are shown in Figure 2.

Correlations with Symptom Measures

In the ASD group there were no significant correlations between GSH concentrations in either voxel and scores on the ADOS, the ADI, the BDI, the STAI and the BSSR scales.

Tissue Composition and Data Quality

There was no significant group difference in percentage of grey matter, white matter or CSF in either voxel. Figure 3 shows an example of [1H]MRS spectrum after LCModel 6.3-0I fitting.

Table I. Participant Characteristics

Characteristic		ASD Patients	Healthy controls
Age		31.82 (8.63)	26.94 (7.39)
ADOS	Social behavior	16.47 (5.97)	Not applicable
	Communication	12.49 (7.26)	Not applicable
ADI	Social behavior	16.47 (5.97)	Not applicable
	Communication	12.49 (7.26)	Not applicable
	Repetitive behavior	4.67 (2.94)	Not applicable
BDI		8.97 (10.23)	3.00 (4.43)
STAI		52.19 (12.90)	35.29 (8.88)
BSSR Childhood scale		6.35 (5.04)	1.95 (2.01)
BSSR Adulthood scale		3.60 (3.62)	1.50 (1.58)

Discussion

This is the first study to quantify glutathione (GSH) brain concentrations *in vivo* in unmedicated adult men with ASD compared to healthy controls. We observed no group differences in GSH concentrations in either the basal ganglia or the dorsomedial prefrontal cortex and no relationship between GSH levels and clinical symptoms.

Our finding contrasts with prior investigations that have reported decreased GSH in blood plasma in ASD [James et al., 2004], however, it is consistent with post-mortem evidence reporting no difference in GSH in the frontal cortex of individuals with ASD [Chauhan et al., 2012]. Thus, our findings do not support the hypothesis that neuronal damage, secondary to oxidative stress caused by deficient GSH contributes to ASD pathogenesis [Deth et al., 2008; Main et al., 2012].

We emphasize that our study included only right handed males with normal intelligence. Therefore, it remains possible that GSH abnormalities exist in other subgroups of individuals with ASD, for example, women, or those with intellectual disability. Age may be especially important to consider in this context because GSH levels have been reported to vary in other age groups [Currais & Maher, 2013; Maurya & Rizvi, 2010]. For example, prior investigations have found that GSH is significantly lower in children with ASD [Chauhan et al., 2012; Frustaci et al., 2012; James et al., 2004]. Future longitudinal studies are needed to address this.

Alternatively, it is possible that a difference in GSH concentrations does exist in the population that we studied, but that we were unable to detect it (Type II

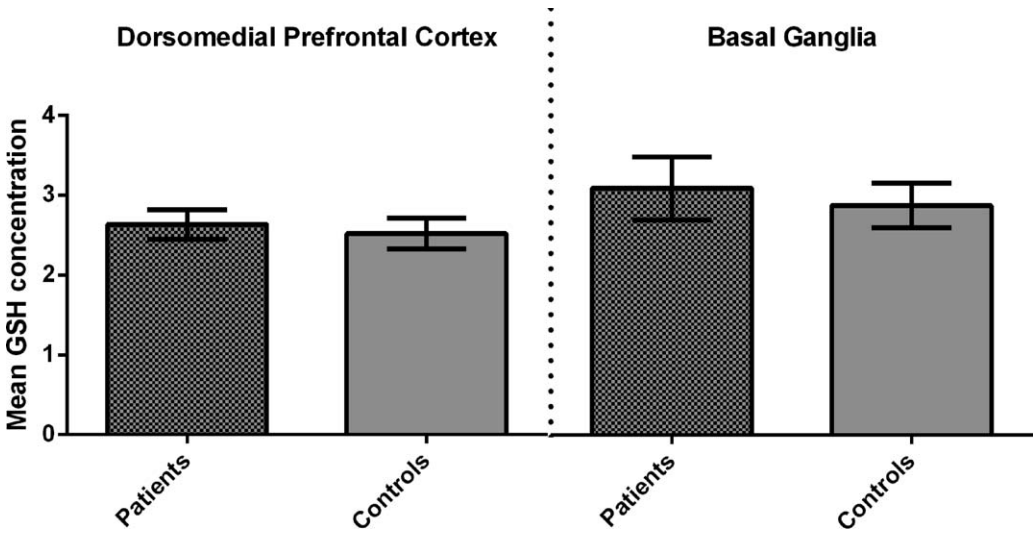

Figure 2. Mean corrected glutathione concentrations in the dorsomedial prefrontal cortex and the basal ganglia did not differ significantly between groups. Error bars represent the standard error of the mean. (GSH, Glutathione; BG, Basal Ganglia; DMPFC, Dorsomedial Prefrontal Cortex).

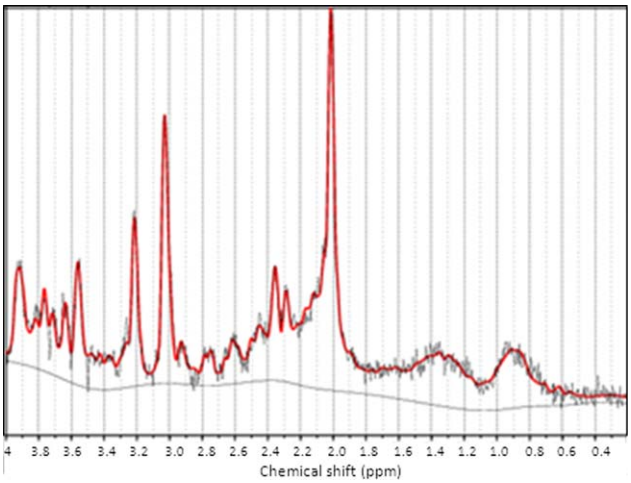

Figure 3. Example of a proton magnetic resonance spectroscopy spectrum after LCModel 6.3-0I fitting.

error). Our sample size was modest, reflecting our decision to limit our sample to those individuals who were not taking any medications that might affect brain chemistry. However, our sample size was similar to that of Wood et al. [2009] who found a robust 22% difference in GSH concentrations at threshold of $P < 0.05$ using similar approaches in individuals with schizophrenia, and larger than that of Godlewska et al. [2014] who found no difference in GSH concentrations between bipolar participants and healthy controls. In addition, a post hoc power calculation based on the present data confirmed that if any group differences do exist these are likely to be extremely small and therefore arguably trivial; as a total sample size of at least $n = 1200$ would be necessary for detection (80% power, at $P < 0.05$).

Our study has a number of other limitations. We did not exclude participants with co-morbid anxiety, depression and ADHD symptoms, but we measured these symptoms using validated clinical scales. We found that ASD participants had higher levels of symptoms than controls. However this was not unexpected. These are very common comorbidities in ASD and a sample without any comorbid disorders would be quite unrepresentative for the spectrum [Mazzone et al., 2012].

From a methodological perspective, GSH is often quantified using a J-edited MEGAPRESS sequence with a longer echo time (TE), but we employed a short TE PRESS sequence to quantify GSH. However, others have shown that GSH can be measured using PRESS at similar echo times [Lagopoulos et al., 2013; Wood et al., 2009]. While the test-retest reliability of PRESS GSH estimates have never been specifically investigated, PRESS is known to provide reliable quantification for other common metabolites [Fayed, Modrego, & Medrano, 2009].

A further consideration is that we investigated only the basal ganglia and the dorsomedial prefrontal cortex and differences in GSH might yet be found in other brain areas. For example, lower levels of GSH in ASD have been reported in the temporal lobe and cerebellum postmortem [Chauhan et al., 2012]. Indeed, concentration differences in other brain metabolites are known to be regionally specific in ASD [Horder et al., 2013]. Finally, we did not measure the oxidised counterpart of GSH, GSSG. Hence we cannot exclude the possibility that GSSG levels are altered in our ASD sample, resulting in GSH redox imbalance.

In summary, the data presented here suggest that oxidative stress resulting from low levels of GSH is not a feature of adult men with normal IQ and ASD. However, we cannot exclude the possibility that further exploration of GSH in other subgroups of ASD (for example, those with low IQ), and at different ages, may help identify individuals who may benefit from antioxidant treatments.

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