Increased prefrontal GABA concentrations in adults with autism spectrum disorders

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Abstract
The excitatory-inhibitory imbalance hypothesis postulates dysregulation of the gamma-aminobutyric acid (GABA) and glutamate (Glu) neurotransmitter systems as a common underlying deficit in individuals with autism spectrum disorders (ASD). Previous studies suggest an important role of these systems in the pathophysiology of ASD, including a study of our group reporting decreased glutamate concentrations in the pregenual anterior cingulate cortex (ACC) of adults with ASD. The aim of this study was to replicate our previous findings of impaired glutamate metabolism in ASD in a new sample and to additionally quantify GABA in the ACC and dorsolateral prefrontal cortex (dlPFC). Concentrations of GABA and glutamate-glutamine (Glx; combined glutamate and glutamine signal) were quantified in the ACC and dlPFC of 43 adults with ASD and 43 neurotypical controls (NTC) by magnetic resonance spectroscopy (MRS). The ASD group showed increased absolute GABA concentrations and elevated GABA/creatine ratios in the left dlPFC compared to NTC, while no group differences were detected in the pregenual and dorsal ACC. Previous findings of altered Glx concentration in the pregenual ACC of the ASD group could not be replicated. Regarding Glx concentrations and Glx/creatine ratios, there were no significant differences in the dlPFC and ACC either. The study supports the hypothesis of an altered GABA and glutamate equilibrium, indicating an imbalance between excitatory and inhibitory metabolism in ASD patients. However, inconsistent results across studies and brain regions suggest a complex underlying phenomenon.

Lay Summary:
• Adults of the autism spectrum exhibit elevated levels of the inhibitory neurotransmitter GABA in the left dorsolateral prefrontal cortex.
• This finding supports the hypothesis of an imbalance between excitatory and inhibitory equilibrium in patients with autism spectrum disorders.

KEYWORDS
anterior cingulate cortex, autism spectrum disorder, dorsolateral prefrontal cortex, GABA, gamma-aminobutyric acid, glutamate
INTRODUCTION

Autism spectrum disorders (ASD) are characterized by deficits in social communication and interaction as well as repetitive behavioral patterns (Levy et al., 2009). ASD has a multifactorial genesis with a strong genetic component, but still a largely unresolved etiology (Nordenbaek et al., 2014; Rosenberg et al., 2009).

An imbalance between excitatory and inhibitory neuronal functioning has been proposed as a common underlying deficit of multiple converging etiologies in some ASD patients (Rubenstein & Merzenich, 2003). This theory states that a shift of synaptic excitation and inhibition is associated with abnormal glutamatergic and GABAergic (gamma-aminobutyric acid) metabolism (Tebartz van Elst et al., 2014a, 2014b; Uzunova et al., 2016), resulting in increased noise and hyperexcitability in the cerebral cortex (Rubenstein & Merzenich, 2003).

1H-MRS (Magnetic Resonance Spectroscopy) studies have investigated the hypothesis of an altered glutamine-glutamate-GABA system in patients with ASD (Ajram et al., 2019). MRS is a noninvasive imaging technique that generates a frequency spectrum by exploiting the nuclear magnetic resonance properties of hydrogen atoms. Metabolites can be identified by the position of their signal peak, the so-called chemical shift (Ajram et al., 2019; de Graaf, 2000). Part of the GABAergic and glutamatergic metabolism can be directly quantified by MRS in vivo in terms of glutamate (Glu) concentration or the joint concentration of Glu + glutamine (Gln) (denoted by Glx), and GABA concentration (Duarte et al., 2012; Novotny et al., 2003). Single voxel spectroscopy, as applied in this study, measures the spectrum in a specific cuboid region of interest (ROI) of the brain.

Preceding MRS GABA, Glu, and Glx studies in patients with ASD show inconsistent results with region specific trends. So far, the exploration of GABA predominantly revealed decreased GABA concentrations (Ajram et al., 2019) in the frontal (Harada et al., 2011; Kubas et al., 2012), auditory (Gaetz et al., 2014; Port et al., 2017; Rojas et al., 2014) and motor cortices (Gaetz et al., 2014), the cerebellum and the anterior cingulate cortex (ACC) (Ito et al., 2017) as well as sensorimotor areas especially of children, adolescents (Puts et al., 2017), but also adults (Sapey-Triomphe et al., 2019) with ASD. However, some studies failed to detect any significant difference compared to neurotypical controls (NTC). These studies comprised the cerebellum (Goji et al., 2017) and the ACC (Brix et al., 2015; Cochran et al., 2015; Goji et al., 2017) of children, the occipital cortex (Drenthen et al., 2016; Puts et al., 2017) of children, the prefrontal and frontal cortices of both children (Carvalho Pereira et al., 2018) and adults (Ajram et al., 2017; Horder et al., 2018; Kirkovski et al., 2018) as well as the motor cortex of adults (Kolodny et al., 2020; Robertson et al., 2016) with ASD. A recent study found no differences in GABA_A receptor densities, but higher GABA/water in the left dorsolateral prefrontal cortex (dLPFC) of adults with ASD (Fung et al., 2021).

Based on the observation of an increased prevalence of seizures in ASD which was explained by neuronal hyperexcitability (Tuchman et al., 2010), an imbalance of excitatory and inhibitory neurotransmission has been postulated as a common underlying deficit of ASD (excitation/inhibition [E/I]) imbalance theory (Rubenstein & Merzenich, 2003). According to the E/I imbalance theory, this imbalance between excitatory and inhibitory neurotransmission is reflected in an increase in glutamatergic (excitatory) and a decrease in GABAergic (inhibitory) signaling (Rubenstein, 2010; Rubenstein & Merzenich, 2003). However, with respect to glutamate, both a hypoglutamatergic and a hyperglutamatergic theory has been proposed (Eltokhi et al., 2020; Sapey-Triomphe et al., 2021). Based on animal studies in which hypoglutamatergic animals exhibited autistic behavior, the hypoglutamatergic hypothesis of ASD has been postulated (Carlsson, 1998). The hyperglutamatergic hypothesis was supported by increased concentrations of plasma glutamate in patients with ASD (Zheng et al., 2016). However, both hypotheses can be reconciled with the disturbed E/I equilibrium theory, considering that both hyperglutamatergic and hypoglutamatergic states lead to a disturbed homeostatic equilibrium (Tebartz van Elst et al., 2014a, 2014b).

MRS investigations detected increased Glu or Glx concentrations (Adults: amygdala-hippocampal complex (Page, 2006), inferior frontal gyrus (Sapey-Triomphe et al., 2021), and auditory cortex (Brown et al., 2013); children: sensorimotor cortex (He et al., 2021), ACC (Beijani et al., 2012; Hassan et al., 2013; Joshi et al., 2013; Naaijen et al., 2017), frontal lobe (Hassan et al., 2013), and putamen (Doyle-Thomas et al., 2014), while other studies found decreased levels (Adults: ACC (Bernardi et al., 2011; Tebartz van Elst et al., 2014a, 2014b), basal ganglia (Horder et al., 2013; Horder et al., 2018); children: cerebellum (DeVito et al., 2007), frontal lobe (Kubas et al., 2012), thalamus (Hegarty et al., 2018)) or no significant differences (e.g. Adults: frontal regions (Ajram et al., 2017; Endres et al., 2015; Horder et al., 2013, 2018), ACC (Endres et al., 2017; Libero et al., 2015, 2016), thalamus (Bernardi et al., 2011); children: medial prefrontal cortex (Carvalho Pereira et al., 2018), ACC (Brix et al., 2015; Cochran et al., 2015; Goji et al., 2017; Ito et al., 2017), caudate or thalamus (Doyle-Thomas et al., 2014)). A recent investigation in adults with ASD reported elevated ratios of Glx/GABA+ in the right inferior frontal gyrus being associated with reduced predictive abilities as well as increased Glx levels in this region (Sapey-Triomphe et al., 2021). The variability of previous findings may be due to differences in study samples (e.g., age groups, inclusion criteria, and random group composition), brain regions investigated, in 1H-MRS sequences (e.g., STEAM, PRESS, and MEGA-PRESS) or the scanner (1.5 Tesla, 3 Tesla) (Ajram et al., 2019).
Based on prior investigations summarized above and theoretical considerations, the dorsal anterior cingulate cortex (dACC), the pregenual anterior cingulate cortex (pACC) and the dlPFC seem to be promising ROIs for further empirical research in this field.

The anterior cingulate cortex (ACC) is implicated in the regulation of emotional and cognitive processes (Bush et al., 2000), with dorsal portions (dACC) being involved in the control of adequate behavior and the processing of complex cognitive tasks (Agam et al., 2010; Di Martino et al., 2009; Heilbronner & Hayden, 2016), and the posterior part (pACC) in the processing of emotions and motivational information (Mao et al., 2017). Patients with ASD showed a glucose hypometabolism in the ACC and a reduced volume of the right anterior cingulate gyrus (Haznedar et al., 2000). Furthermore, in the ACC of patients with ASD, a decreased density of GABA_B receptors was detected in one study (Oblak et al., 2010).

Earlier 1H-MRS studies focusing on the ACC of adults with ASD showed decreased Glu or Glx levels (Bernardi et al., 2011; Tebartz van Elst et al., 2014a, 2014b) or no significant group differences (Endres et al., 2015; Libero et al., 2015, 2016). MRS investigations detected reduced GABA concentrations in the ACC of children with ASD (Ito et al., 2017) whereas other studies reported no difference in the ACC compared to the NTC group (Brix et al., 2015; Cochrane et al., 2015; Goji et al., 2017).

The dorsolateral prefrontal cortex (dlPFC) represents a conflict monitoring loop with the ACC and thus plays an important role in complex metacognitive processes (Solomon et al., 2014). In addition, the left dlPFC is responsible for the so called top-down control (Fassbender et al., 2006) and especially was reported to be relevant for the recognition of emotions in faces (Balconi & Canavesio, 2016).

An involvement of the dlPFC in ASD has also been suggested by structural magnetic resonance imaging (MRI) studies, which found increased gray matter (GM) volume in this area in adults with ASD (Ecker et al., 2012). A reduced activation of the dlPFC in patients with ASD while performing spatial working memory tasks (Luna et al., 2002) was reported. A decreased expression of the metabotropic glutamate receptor 5 (mGluR5) was found in the dlPFC of patients with ASD (Chana et al., 2015).

MRS studies in adults with ASD reported no significant GABA concentration differences (Ajram et al., 2017; Horder et al., 2018; Kirkovski et al., 2018) or increased GABA levels (Fung et al., 2021) in frontal and prefrontal areas compared to NTC. With regard to Glu or Glx concentrations, increased (Sapey-Triomphe et al., 2021) as well as similar Glu or Glx concentrations (Ajram et al., 2017; Endres et al., 2015; Horder et al., 2013, 2018) have been reported in the frontal or prefrontal cortex of adults with ASD.

The aim of this study was to (I) replicate our previous finding of decreased Glx concentrations in the pACC of adults with ASD (Tebartz van Elst et al., 2014a, 2014b) in a new sample and to (II) now quantify GABA in addition to Glx in this and other brain areas (dACC and dlPFC) applying a MEGA-PRESS MRS protocol to investigate the role of the inhibitory GABAergic system in ASD.

Based on preceding studies in adults, our own previous study (Tebartz van Elst et al., 2014a, 2014b) and the afore mentioned function of the investigated areas for autism research, we expected reduced Glx and GABA signals in patients with ASD compared to NTC.

METHODS

Participants

The study was approved by the local ethics committee of the University Medical Center Freiburg (Approval ID: 253/12). We investigated N = 43 patients (33 male, 10 female) with ASD and N = 43 (30 male, 13 female) NTC. The age of the ASD group was 34.1 ± 11.7 (18–55 years) while that of the NTC was 34.8 ± 10.7 (20–61 years).

The patients were recruited from the Department of Psychiatry and Psychotherapy and the Department of Child and Adolescent Psychiatry of the University Medical Center Freiburg.

The diagnosis was established by experienced psychiatrists and psychologists according to the International Classification of Diseases (ICD-10) criteria for Asperger syndrome (F84.5) and the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) criteria for ASD (299.00). The diagnostic process was based on the recommendations of the National Institute for Health and Clinical Excellence (NICE) for adult autism (https://www.nice.org.uk/guidance/cg142; retrieval date February 28th, 2022). The diagnosis of all ASD patients was confirmed by a certified psychologist applying the ADOS diagnostic tool (Lord et al., 2000). Psychometric tools included the following instruments: the autism spectrum quotient (AQ [Baron-Cohen et al., 2001]), the empathy quotient (EQ [Baron-Cohen & Wheelwright, 2004]) and the Social Responsiveness Scale (SRS2 [Constantino & Gruber, 2007]).

Patients with episodes of bipolar disorder, schizophrenia, borderline personality disorder, antisocial personality disorder, eating disorders (BMI < 18 kg/m²), or neurological diseases with potential effects on the assessment outcome, such as epilepsy, were excluded from the study. Additionally, substance or alcohol abuse, the use of benzodiazepines or sedatives (zopiclone or zolpidem) during the last 12 months were defined as exclusion criteria. Patients with the common comorbidities attention-deficit/hyperactivity disorder (ADHD),...
depression or mild forms of anxiety or obsessive–compulsive disorder were not excluded. Psychiatric comorbidities were assessed applying the Symptom Checklist (SCL-90 [Derogatis & Savitz, 2000]), the Beck Depression Inventory (BDI-II [Beck et al., 1961; Hautzinger et al., 2006]) and the Wender-Utah Rating Scale (WURS-k [Retz-Junginger et al., 2002]).

MRS data were acquired from an independent sample of 50 newly recruited patients with ASD. The reasons for exclusion in the ASD group were the following: Four patients took methylphenidate daily, one had a substance abuse, one suffered from psychotic symptoms and one showed head motion artifacts in the T1-image. Therefore, the final sample comprised 43 patients with ASD (33 male, 10 female). Females were not scanned at a particular phase of their menstrual cycles.

The NTC was matched to the ASD group according to age, sex and intelligence quotient (IQ). In both groups an IQ > 75 was mandatory, which was assessed with the Multiple-choice vocabulary test (MWT-B [Lehrl et al., 1995]) and the Culture Fair Intelligence Test 20—Revision (CFT-20 [Weiß et al., 2006]).

In order to rule out Axis I disorders in NTC participants, a structured clinical interview for DSM-IV (SCID I; [Wittchen et al., 1997]) was conducted with all participants of the control group. Additionally, psychometric tools included the following instruments: the AQ (Baron-Cohen et al., 2001), EQ (Baron-Cohen & Wheelwright, 2004), SRS-2 (Constantino & Gruber, 2007), SCL-90 (Derogatis & Savitz, 2000), BDI-II (Beck et al., 1961; Hautzinger et al., 2006), and the WURS-k (Retz-Junginger et al., 2002).

Forty-five NTC were investigated by MRS, whereby one control had to be excluded due to a recent transient ischemic attack and another one due to MRS data loss. Finally, 43 NTC (30 male, 13 female) were included in the final analysis.

**Data acquisition**

Scanning was performed at the Imaging Center of the Department of Radiology of the University Medical Center Freiburg with a 3-Tesla Prisma MR system (Siemens Healthineers, Germany). The scanner was equipped with a 64-channel head coil for signal reception. First, an anatomical scan was conducted with a T1-weighted magnetization Prepared Rapid Gradient Echo (MPRAGE) protocol (sagittal slice orientation, TR = 2300 ms, TE = 2.98 ms, flip angle = 9°; FOV = 256 × 256 mm², voxel size = 1 × 1 × 1 mm³). The spectroscopic data of three voxels in the dACC (voxel size = 20 × 28 × 25 mm³), the pACC (voxel size = 20 × 20 × 20 mm³) and the dlPFC (voxel size = 25 × 25 × 25 mm³) were obtained using a MEGA-PRESS sequence (TR = 1500 ms, TE = 68 ms, spectral bandwidth = 1200 Hz, 512 spectral averages).

The difference editing method MEGA-PRESS (Mescher et al., 1998) was applied to filter out the GABA signal from the background of uncoupled resonances, mainly arising from Creatine (Cr), on the basis of its specific J-coupling evolution (Mullins et al., 2014; Puts et al., 2017). Spectroscopic measurements with water suppression were followed by a water unsuppressed reference measurement with 16 averages for absolute metabolite quantification. Water suppression was performed using the water suppression enhanced through T1 effects (WET) technique (Ogg et al., 1994). The dACC voxel was positioned tangentially to the dorsal side of the corpus callosum to include equal parts of the dACC of both hemispheres. The voxel locations are illustrated in Figure 1.

The pACC voxel was placed in a rostral position of the corpus callosum. The localization and size were in line with the placement of the pACC voxel in our previous investigation (Tebartz van Elst et al., 2014a, 2014b). The third voxel was positioned in the dlPFC of the left hemisphere. The aim was to achieve a maximum proportion of GM with a minimum amount of cerebrospinal fluid (CSF) and to avoid contact with the meninges, which give rise to macromolecular resonances in the spectral ROI.

We successfully acquired 43/41 dlPFC, 41/43 dACC, and 34/34 pACC spectra for ASD and NTC participants, respectively. Reporting of the MRS parameters was based on the Minimum Reporting Standards for in vivo MRS experts’ consensus recommendations (Lin et al., 2021).

**Data analysis**

Tissue segmentation of the acquired MPRAGE dataset was performed with SPM12 (Statistical Parametric Mapping, University College London) (Penny et al., 2007), a MATLAB-based software (The MathWorks, Massachusetts, USA) to calculate the percentage of GM, white matter (WM) and CSF for each voxel.

Automatic quantification of the acquired 1H-MRS spectra was performed with LCModel based on the linear combination of numerically simulated metabolite basis spectra (Provencher, 2001). Absolute metabolite concentrations were determined using the internal water reference method and applying relaxation correction with relaxation constants obtained from the literature (Near et al., 2021). Frequency and phase correction of the individual spectral averages was performed retrospectively using the Gannet software (Edden et al., 2014). Because Glu and Gln are poorly resolved with a GABA-optimized MEGA-PRESS sequence, we report the Glu and Gln combined as Glx. All spectra with a signal-to-noise Ratio (SNR) of less than 5 (as determined by LCModel) were excluded from further analysis. For insufficient SNR (<5) in the spectra,
five ASD participants were excluded from the GABA dIPFC and Glx dIPFC analyses. The following data were considered for the final statistical analyses of GABA: 38 ASD/41 NTC for the dIPFC voxel, 41 ASD/43 NTC for the dACC and 34 ASD/34 NTC for the pACC voxel. Glx signals of 38 ASD/41 NTC for the dIPFC voxel, 41 ASD/43 NTC for the dACC and 34 ASD/34 NTC for the pACC voxel were included in the final analysis.

Supplemental Table 1 (“Data quality table”) reports full width at half maximum (FWHM), SNR, Cramer-Rao Lower Bounds (CRLBs), frequency standard deviation as determined from the retrospective frequency drift correction, partial GM, WM and CSF volumes of the ASD and NTC group.

The raw GABA and Glx concentrations (institutional units = IU) were corrected for the partial CSF compartment and differences in metabolite concentrations between GM and WM (alpha tissue correction) according to Harris et al. (2015). For GABA, an alpha factor of 0.43 based on Harris et al. (2015), for Glx an alpha of 0.85, and for Cr of 0.91, both based on Zheng et al. (2016) was used. For purposes of comparison, we calculated the corresponding alpha factors across all voxels from the data of this study. The following alpha values could be determined: GABA = 0.50 (ASD = 0.74, NTC = 0.32), Glx = 0.68 (ASD = 0.60, NTC = 0.75), Glu = 0.75 (ASD = 0.60, NTC = 0.91), Cr = 0.86 (ASD = 0.80, NTC = 0.92). The group tissue corrected metabolite concentrations were group normalized to the average GM and WM fractions. GABA/Cr and Glx/Cr ratios were calculated from the tissue-corrected GABA and Glx concentrations divided through the tissue-corrected Cr ratios.

**Statistical analyses**

The statistical analysis was performed with R (The R Foundation for Statistical Computing, Vienna, Austria) (R Core Team, 2021).

Comparability of the ASD and control group in terms of age, IQ and sex were verified by two-sample t-tests and Chi-square tests by Pearson, respectively. Since the voxels’ partial GM and WM volumes significantly explained variance of the tissue-corrected and group normalized metabolite concentrations, we adjusted the tissue-corrected and group normalized metabolite concentrations for differences in partial GM and WM volume (Ding et al., 2016; Gao et al., 2013; Grachev & Apkarian, 2000) together with age, sex, and SNR using a linear model (the “predict” function of the R stats package). All further metabolite statistics were performed with these adjusted, tissue-corrected and group
A potential difference in GABA concentration between the two groups was determined by a robust independent sample t-test (Wilcox, 2012) with bootstrapping (Yuen’s test for trimmed means with bootstrap “yuenbt” with 10,000 samples) of the WRS2 package (Mair & Wilcox, 2020), as not all metabolite concentrations were normally distributed. Robust measures of effect sizes \( \xi \) were calculated with \( \xi \)-values of 0.10, 0.30, and 0.50 corresponding to small, medium, and large effect sizes, respectively (Wilcox & Tian, 2011). The same procedure was performed for Glx. The significance level for multiple comparisons was determined by false-discovery rate correction (FDR). The significance level was set at \( p < 0.05 \).

For reasons of comparability with some earlier studies, we also report the GABA/Cr and Glx/Cr ratios, applying the procedure described for the absolute metabolite concentrations.

A potential linear correlation between the tissue-corrected, group-normalized and adjusted (age, sex, partial volume and SNR) GABA and Glx concentration levels and the psychometric results of the AQ (Baron-Cohen et al., 2001), EQ (Baron-Cohen & Wheelwright, 2004), ADOS (Lord et al., 2012), SRS-2 (Constantino & Gruber, 2007), BDI-II (Beck et al., 1961; Hautzinger et al., 2004), or symptoms of an ADHD in childhood based on the WURS-k (Retz-Junginger et al., 2002) and CFT 20-R (Weiß et al., 2006) were examined with robust correlations “pbcor” of the WRS2 package (Mair & Wilcox, 2020). Results were corrected for multiple comparison using FDR, separately for the ASD group and both groups combined, as well as the different questionnaires.

To determine the effect of potential interfering factors, the data were additionally adjusted for the influence of nicotine consumption, comorbidities and medication intake before testing the group comparisons mentioned above. For this purpose, the medication was pooled in the two clusters of antidepressants and antipsychotics. In terms of comorbidities, the influence of depression and ADHD on the group statistics was investigated.

### RESULTS

#### Demographic and psychometric data

The ASD and the NTC group did not differ significantly in terms of sex, age or IQ. All subjects had an IQ > 93 with an average IQ of 117 (Table 1). The ASD and the NTC groups showed significant differences in their depressive symptomatology according to the BDI-II questionnaire (Beck et al., 1961; Hautzinger et al., 2006) or symptoms of an ADHD in childhood based on the WURS-k (Retz-Junginger et al., 2002). The GM, WM, total brain, total intracranial or CSF volumes did not differ between the ASD and NTC groups (Table 1). Eighteen participants with ASD received at least one

### TABLE 1

Demographic, psychometric and brain segment volume data of the ASD and NTC group (\( p \)-values derived from two-sample \( t \)-tests for continuous and \( \chi^2 \)-tests for categorical variables)

|                  | ASD \((N = 43)\) | NTC \((N = 43)\) | \( p \)-value* | \( t \)-values, \( \chi^2 \) |
|------------------|-----------------|-----------------|---------------|--------------------------|
| **Age** (mean ± SD) | 34.1 ± 11.7 | 34.8 ± 10.7 | 0.781 | −0.279 |
| **Sex** (male/female) | 33/10 | 30/13 | 0.626 | 0.23741 |
| IQ-CFT20-R | 117.3 ± 24.1 | 117.4 ± 17.6 | 0.98 | −0.026 |
| IQ-MWT-B | 117.0 ± 14.5 | 117.3 ± 12.7 | 0.937 | −0.079 |
| SRS2 | 101.1 ± 32.0 | 33.4 ± 16.9 | <0.001 | 12.271 |
| AQ | 35.0 ± 8.8 | 13.2 ± 7.0 | <0.001 | 12.681 |
| EQ | 23.3 ± 13.0 | 49.2 ± 12.5 | <0.001 | −9.431 |
| BDI-II | 19.1 ± 14.8 | 5.3 ± 6.8 | <0.001 | 5.548 |
| WURS-k | 29.0 ± 14.3 | 11.2 ± 11.5 | <0.001 | 6.338 |
| GM (ml) | 731.0 ± 75.8 | 725.8 ± 68.1 | 0.742 | 0.33 |
| WM (ml) | 544.4 ± 59.7 | 551.6 ± 68.3 | 0.605 | −0.519 |
| CSF (ml) | 383.5 ± 50.3 | 379.4 ± 50.6 | 0.705 | 0.379 |
| TBV (ml) | 1275.4 ± 122.3 | 1277.4 ± 126.1 | 0.939 | −0.076 |
| TIV (ml) | 1658.9 ± 135.2 | 1656.8 ± 150.5 | 0.946 | 0.067 |

*Note: Significant \( p \)-values are depicted in bold letters.
*Uncorrected \( p \)-values.

Abbreviations: AQ, autism-spectrum quotient (Baron-Cohen et al., 2001); ASD, autism spectrum disorder; BDI-II, Beck Depression Inventory II (Beck et al., 1961; Hautzinger et al., 2006); EQ, empathy quotient (Baron-Cohen & Wheelwright, 2004); GM, gray matter; IQ-CFT20-R, intelligence quotient assessed with the culture fair test (Weiß et al., 2006); IQ-MWT-B, intelligence quotient assessed with the multiple choice vocabulary test (Lehrl et al., 1995); N, number; NTC, neurotypical control; SD, standard deviation; SRS, Social Responsiveness Scale (Constantino & Gruber, 2007); TBV, total brain volume; TIV, total intracranial volume; WM, white matter; WURS-k, Wender-Utah rating scale (Retz-Junginger et al., 2002).
Table 2: Adjusted tissue corrected IU and Cr-ratios of three brain areas (dIPFC, dACC and pACC) of the ASD and NTC group. Adjusted metabolite concentrations were corrected for linear influences of age, sex, SNR, partial GM, and WM volume.

| Metabolite | Region  | Institutional units | Cr-ratios |
|------------|---------|---------------------|-----------|
| GABA       | dIPFC   | ASD (mean ± SD)     | NTC (mean ± SD) |
|            |         | 3.45 ± 0.93         | 2.67 ± 0.91 |
|            |         | <0.001              | 0.61 ± 0.19 |
| GABA       | dACC    | 3.73 ± 1.15         | 3.96 ± 1.03 |
|            |         | 0.328               | 0.35 ± 0.08 |
| GABA       | pACC    | 5.5 ± 1.37          | 5.5 ± 1.28 |
|            |         | 0.994               | 0.62 ± 0.16 |
| GABA       | dACC    | 8.19 ± 1.69         | 8.48 ± 1.26 |
|            |         | 0.396               | 0.93 ± 0.18 |
| GABA       | pACC    | 11.57 ± 2.56        | 12.16 ± 1.80 |
|            |         | 0.229               | 1.1 ± 0.12 |
| GABA       | dACC    | 8.85 ± 2.23         | 8.10 ± 1.80 |
|            |         | 0.132               | 0.97 ± 0.18 |
| GABA       | pACC    | 1.03 ± 0.328        | 0.35 ± 0.37 |
|            |         | 0.002               | 0.48 ± 0.17 |
| Glx        | dIPFC   | 0.91 ± 0.238        | 0.19 ± 0.48 |
|            |         | 0.002               | 0.61 ± 0.19 |
| Glx        | dACC    | 1.80 ± 0.132        | 1.15 ± 0.95 |
|            |         | 0.229               | 1.1 ± 0.12 |
| Glx        | pACC    | 2.23 ± 0.57         | 1.69 ± 0.99 |
|            |         | 0.132               | 0.97 ± 0.18 |

Note: Significant p-values are depicted in bold letters.
*FDR correction across GABA and Glx for all three voxels as part of the main hypothesis.

Abbreviations: ASD, autism spectrum disorder; Cr, Creatine; dACC, dorsal anterior cingulate cortex; dIPFC, dorsolateral prefrontal cortex; FDR, false discovery rate; GABA, gamma-aminobutyric acid; Glx, glutamine and glutamate; GM, gray matter; IU, Institutional Units; NTC, neurotypical controls; pACC, pregenual anterior cingulate cortex; SD, standard deviation; SNR, signal-to-noise ratio; WM, white matter.

Psychiatric medication, 9 took SSRI/SNRIs, 9 took other antidepressants, two low-dose neuroleptics as sleeping pills, and 2 stimulants not regularly (methylphenidate, modafinil).

MRS results

In the dIPFC, significantly higher GABA concentrations were observed in the ASD compared to the NTC group (t[NA] = 3.20, \( p_{uncorr} = 0.002 \), \( p_{FDR} = 0.010 \), \( \xi = 0.514 \), Table 2, Figure 1) after adjustment of data for age, sex, SNR, partial GM and WM.

In the dACC (t[NA] = −0.73, \( p_{uncorr} = 0.443 \), \( p_{FDR} = 0.665 \), \( \xi = 0.132 \)) and the pACC (t[NA] = 0.16, \( p_{uncorr} = 0.857 \), \( p_{FDR} = 0.868 \), \( \xi = 0.031 \)), there was no significant difference in GABA concentrations.

Neither in the dIPFC (t[NA] = −0.16, \( p_{uncorr} = 0.868 \), \( p_{FDR} = 0.868 \), \( \xi = 0.025 \)) nor in the pACC (t[NA] = 1.10, \( p_{uncorr} = 0.250 \), \( p_{FDR} = 0.501 \), \( \xi = 0.213 \)) a significant difference in the Glx concentration could be detected between the two groups.

The GABA/Cr ratio in the dIPFC was also significantly higher in the ASD group (t[NA] = 2.69, \( p_{uncorr} = 0.008 \), \( p_{FDR} = 0.047 \), \( \xi = 0.446 \), Table 2, Figure 2).

The dIPFC Glx/Cr ratios, however, were not significantly different between the ASD and the NTC groups (t[NA] = −1.57, \( p_{uncorr} = 0.109 \), \( p_{FDR} = 0.327 \), \( \xi = 0.258 \), Figure 2), as neither of the other voxels showed significant differences in GABA/Cr or Glx/Cr ratios.

N-Acetylaspartic acid (NAA), Creatine, glycerylphosphorylcholine (GCP) concentrations and the Cr-ratios did not significantly differ in the investigated voxels (dIPFC, dACC, pACC) between the ASD and NTC group (Supplemental Table 2).

Correction for further confounding effects

After adjustment for the influence of nicotine consumption (t[NA] = 3.18, \( p = 0.003 \)), depressiveness (t[NA] = 2.28, \( p = 0.028 \)), comorbid ADHD symptoms in childhood ((WURS-k) (Retz-Junghöfer et al., 2002) (t[NA] = 2.14, \( p = 0.040 \)), antidepressant (t[NA] = 2.05, \( p = 0.042 \)) or antipsychotic medication (t[NA] = 2.48, \( p = 0.016 \)), the group difference of the GABA signal in the dIPFC remained significant.

Correlations

We found a significant correlation between measures of cognitive empathy in terms of the EQ (Baron-Cohen & Wheelwright, 2004) with the GABA concentrations in the dIPFC across both study groups, which, however, did not survive correction for multiple testing using FDR. No other metabolite and voxel showed a significant correlation with the AQ (Baron-Cohen et al., 2002), BDI-II (Beck et al., 1996), SRS-2 (Constantino & Gruber, 2007), ADOS scores (Lord et al., 2012), BDI-II (Beck et al., 1996; Hautzinger et al., 2006), WURS-k (Retz-Junghöfer et al., 2002) and CFT 20-R (Weiß et al., 2006), respectively after correction for multiple testing (Figure 3).

Discussion

In contrast to our hypotheses, we (I) could not replicate earlier results of decreased Glx in the pACC in adults with ASD and (2) observed an increased GABA concentration (instead of decreased GABA) and an increased GABA/Cr ratio in the left dIPFC for the ASD compared to the control group, while no significant differences in Glx concentrations or Glx/Cr ratios were detectable in...
this brain area. Neither Glx nor GABA differed between the two groups in the dACC or the pACC.

Considering the previous literature, the increase of the GABA concentration in the dlPFC was not expected in patients with ASD compared to NTC. 1H-MRS studies in children/adolescents showed a lower GABA concentration (Harada et al., 2011; Kubas et al., 2012) or no significant differences (Carvalho Pereira et al., 2018) in the prefrontal and frontal cortices of the ASD group. However, investigations in adults mostly showed no differences in GABA concentration in (pre-)frontal regions (Ajram et al., 2017; Horder et al., 2018; Kirkovski et al., 2018), while one previous study with a similar dlPFC voxel location reported in line with our study increased GABA/water (Fung et al., 2021). Hence, decrease in (pre)frontal GABA concentrations was only reported in children, while adults exhibited increased prefrontal GABA. Nevertheless, most previous studies focused on children with ASD, and it is still unclear to what extent cerebral structures and neurotransmitter ratios in ASD are age-dependent.

Various studies showed an excessive brain volume overgrowth in children with ASD (Courchesne et al., 2005, 2011; Hazlett et al., 2011). Whether this might explain relatively reduced GABA concentrations in childhood remains unresolved. In the current sample, the total brain volume of the NTC and the ASD group did not differ significantly (Table 1).

In line with preceding investigations in children, no significant differences of GABA levels were detected in the ACC (Brix et al., 2015; Cochran et al., 2015; Goji et al., 2017) between patients with ASD and NTC. However, another study (Ito et al., 2017) detected reduced GABA levels in children.

Studies focusing on Glx or Glu showed divergent results with increased (Bejjani et al., 2012; Hassan

**Figure 2** Relative GABA/Cr and Glx/Cr ratios concentrations in the dlPFC, dACC and pACC voxel (adjusted for the effects of age, sex, SNR, partial GM, and WM volume). Box plots show median and confidence intervals (notches) and upper and lower quartile for the ASD (dark blue) and NTC group (light blue). Red line depicts the group mean. Abbreviations: ASD, autism spectrum disorder; Cr, creatine; dACC, dorsal anterior cingulate cortex; dlPFC, dorsolateral prefrontal cortex; GABA, gamma-aminobutyric acid; Glx, glutamine and glutamate; GM, gray matter; NTC, control participants; pACC, pregenual anterior cingulate cortex; SNR, signal-to-noise ratio; WM, white matter.
Figure 3 (a) Uncorrected p-values of the correlation of psychometric scales for ASD (AQ, EQ, SRS-2, ADOS), IQ (CFT 20-R), depression (BDI-II) and ADHD (WURS-k) with adjusted tissue-corrected GABA and Glx concentrations (IU) in the three voxels (dLPFC, dACC and pACC) across the ASD group and both groups. Color coded robust measure of effect size ξ (Wilcox & Tian, 2011; ξ = 0.15, 0.35, and 0.50, corresponding approximately to the small, medium, and large effect sizes). No correlation survived correction for multiple testing. (b) Correlation plot of the EQ-score and the GABA concentration (IU) in the dLPFC across both groups. ADHD, attention-deficit/hyperactivity disorder; ADOS, autism diagnostic observation schedule (Lord et al., 2000); AQ, autism-spectrum quotient (Baron-Cohen et al., 2001); ASD, autism spectrum disorder; BDI, Beck Depression Inventory (Beck et al., 1961; Hautzinger et al., 2006); CFT20-R, Culture Fair Intelligence Test (Weiß et al., 2006); dACC, dorsal anterior cingulate cortex; dLPFC, dorsolateral prefrontal cortex; EQ, empathy quotient (Baron-Cohen & Wheelwright, 2004); GABA, gamma-aminobutyric acid; Glx, glutamine and glutamate; NTC, neurotypical controls; pACC, pregenual anterior cingulate cortex; SRS, Social Responsiveness Scale (Constantino & Gruber, 2007); WURS, Wender-Utah Rating Scale (Retz-Junginger et al., 2002)
et al., 2013; Joshi et al., 2013; Naaijen et al., 2017), decreased (Bernardi et al., 2011; Tebartz van Elst et al., 2014a, 2014b) or no level differences (Endres et al., 2017; Libero et al., 2015, 2016) in the ACC. Nevertheless, it should be considered that some samples were very small, so the statistical validity of these studies might be limited (Bejjani et al., 2012; Bernardi et al., 2011; Cochran et al., 2015; Hassan et al., 2013; Joshi et al., 2013).

Additionally, subjects with intellectual disabilities were included (Hassan et al., 2013) or the groups were not matched for sex and IQ (Bejjani et al., 2012).

In a previous study by our research group on altered glutamate concentrations in ASD using an older PRESS MRS sequence, that does not allow quantification of GABA, lower Glx concentrations were detected in the pACC (Tebartz van Elst et al., 2014a, 2014b). Therefore, the same localization was chosen for this project, but with slightly different dimensions of the voxel, as an increased susceptibility to artifacts induced by sinusoidal air enclosures could be observed in the new setup (PRISMA system). The use of a 64-channel instead of a 12-channel head coil improved SNR. However, in the preceding study a shorter echo time of 30 ms was applied, which may be more suitable for the acquisition of the Glx signal than a TE of 68 ms optimized for GABA detection via MEGA editing (Mescher et al., 1998; Tebartz van Elst et al., 2014a, 2014b). The methodological differences represent possible reasons for the differing findings.

Contrary to the excitatory and inhibitory imbalance hypothesis, but in line with Fung et al. (2021), elevated GABA concentrations were detected in the dlPFC in the ASD group. It is conceivable that in ASD, hyperexcitation in the brain, as described in the excitatory and inhibitory imbalance hypothesis, leads to excessive inhibition in certain areas, which might be reflected by increased GABA concentrations. Such a constellation would relate well to the theory of local-area-network-inhibition (Lani-hypothesis [Tebartz van Elst et al., 2011]) which states that the excessive or unstable excitatory metabolism can induce homeostatic hyper-inhibition, which in turn leads to clinical symptoms, the precise nature of which depends on the involved networks. With this in mind, higher GABA levels have been proposed to represent compensatory mechanisms for altered GABAergic metabolism elsewhere e.g. anomalies in GABA receptor function (Fung et al., 2021; Yip et al., 2008). The left dlPFC is important for top-down control mechanisms (Fassbender et al., 2006; Miller & Cohen, 2001) and the recognition of emotions in faces (Balconi & Canavesio, 2016). Increased inhibition in this area might be responsible for repetitive behavior and inadequate responses due to a lack of top-down control as well as deficits in empathic and social behavior. However, based on the present study, no direct link between the function of the dlPFC and our results can be drawn, as no significant correlation of the psychometric scores with the GABA concentrations in the dlPFC was found.

The inconsistent findings regarding GABA and Glx concentrations in ASD patients may be attributable to different factors. On the one hand, differences in GM/WM/CSF composition (partial volume effects) (Quadrelli et al., 2016) and voxel localization may cause deviations in concentration. In addition, the glutamine-glutamate GABA cycle is a constantly changing system in flux, so that measured transmitter concentrations represent “snapshots” (Ajram et al., 2019).

Further studies investigating GABA concentrations in adults in the left dlPFC are required to establish whether our results of increased GABA concentrations in this brain region are replicable. Moreover, longitudinal studies allow the demonstration of changes in neurotransmitter conditions over time. The fact that the 1H-MRS results of GABA quantification in adults differ from the results in children suggests that neurometabolite profiles are not constant but can vary throughout life.

Limitations

Compared to other studies, slightly smaller voxels were chosen for the detection of neurotransmitters in our approach. The advantage is that a more specific brain region with a more homogeneous tissue composition was examined, so that the result was less influenced by subcutaneous lipid signals. In the pACC voxel, slight inter-individual deviations in localization had to be accepted in favor of homogeneity within the measurement range due to the proximity to the sinus.

The combined quantification of Glu and Gln as Glx instead of pure Glu concentrations can be regarded as a further limitation. For a better separation of Glu and Gln resonances, dedicated sequences and protocols might be applied (Hancu, 2009; Zhang & Shen, 2016). We only reported the more reliable Glx concentrations because the MEGA-PRESS protocol was optimized for GABA and not for Glu quantification. Our analysis pipeline performed a frequency and phase correction, but did not provide any frequency drift output, which would have been helpful in checking for confounding effects from head movement or heating-induced drift issues.

We found a significantly higher frequency standard deviation in the dlPFC voxel of the ASD group (Supplemental Table 1). It should be noted that the applied retrospective frequency and phase correction effectively mitigates subtraction artifacts in the difference spectra, but does not account for drift-induced changes of the GABA+ editing efficiency (Harris et al., 2014). In future studies, this issue might be addressed by using prospective frequency correction as recently proposed by Edden et al. (2016).

Eighteen participants of the ASD group took medication. Previous studies showed that selective serotonin
reuptake inhibitors can reduce glutamate release (Golemboiwksa & Dziubina, 2001) and lead to an increased ratio of GABA/Cr in the occipital lobe (Sanacora et al., 2002). Nevertheless, the group difference in GABA concentration in the dlPFC between ASD and NTC remained significant after we adjusted our statistical model for the effects of antidepressants. For the future, a medication free collective should be examined.

Group differences in dlPFC GABA concentrations persisted even after correction for childhood ADHD symptoms according to the WURS-k questionnaire (Retz-Junginger et al., 2002), which are often comorbidly associated with ASD.

As a further limitation, female participants were not scanned at a particular time during the menstrual cycle. In future studies, it is recommended to take this into account as GABA concentrations change during the menstrual cycle (De Bondt et al., 2015).

SUMMARY

Increased GABA concentrations were found in the left dlPFC in adults with ASD, while there was no difference in Glx concentration. The increased GABA concentration could be interpreted as support for the excitatory-inhibitory imbalance hypothesis, which suggests increased inhibition of the corresponding networks in the dlPFC. Further longitudinal studies of GABA concentrations mapping multiple voxels over the course of the neurodevelopmental disorder are desirable to gain further insights.

AUTHOR CONTRIBUTIONS

Kathrin Nickel, Simon Maier and Ansgard Lena Düppers wrote the paper. Simon Maier, Kathrin Nickel and Ansgard Lena Düppers performed the data and statistical analysis. Ludger Tebartz van Elst, Evgeniy Perlov, Simon Maier, Kathrin Nickel, Dominique Endres organized the study and created the study design. Andreas Riedel, Kathrin Nickel, Thomas Fangmeier, Dominique Endres, Dieter Ebert recruited the patients and established the diagnosis. Ansgard Lena Düppers and Thomas Fangmeier evaluated the psychometric scores. Ansgard Lena Düppers and Simon Maier performed the measurements. Michael Dacko and Thomas Lange implemented the MRS measurement and quantification protocol. Ludger Tebartz van Elst, Evgeniy Perlov, Katharina Domschke, Dieter Ebert, Dominique Endres, Kimon Runge, Michael Dacko, Thomas Lange, Thomas Fangmeier, Andreas Riedel revised the manuscript critically focusing on clinical and statistical aspects. All authors were critically involved in the theoretical discussion and composition of the manuscript. All authors read and approved the final version of the manuscript.

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CONFLICT OF INTERESTS

SM, ALD, KR, MD, TL, TF, AR, DE, DEn, EP, KN have no conflict of interest; KD: member of the “Steering Committee Neurosciences,” Janssen Pharmaceuticals, Inc.; LTvE: Advisory boards, lectures, or travel grants within the last 3 years: Roche, Eli Lilly, Janssen-Cilag, Novartis, Shire, UCB, GSK, Servier, Janssen and Cyberonics.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the first author upon reasonable request.

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